International Symposium

The Dynamics of Virus and Rickettsial Infections

The Symposium was sponsored by the Henry Ford Hospital Detroit Michigan and held at the Hospital October 21, 22, and 23 1953

Program Committee

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International Symposium

The Dynamics of Virus and Rickettsial Infections

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Introduction

An International Symposium on the Dynamics of Virus and Rickettsial Infections was held in Detroit Michigan on October 21 22 and 23 1953 under the auspices of the Henry Ford Hospital This volume constitutes the proceedings of the Symposium and contains the papers that were presented as well as discussions of them

The idea that a Symposium on this broad subject might prove valuable had its origin with the staff of the Henry Ford Hospital An advisory committee composed of Doctors Thomas Francis Jr John G Kidd Joseph E Smadel and Frederick D Stimpert shared with a Ford Hospital committee under the chairmanship of Dr Frank W Hartman and Dr Gerald A LoGrippo the responsibility for the program Thirty three investigators in the field of virus and rickettsial infections were invited to present papers Some traveled from distant parts of the world in order to do so Dr J Ralph Audy for example came from Malaya while Mr F C Bawden Dr A W Downie and Dr F O MacCallum came from England Dr Alfred Gottschalk from Australia and Dr Preben von Magnus from Denmark Other eminent workers came from twenty-one institutions located in various parts of the United States In addition to those who gave papers more than 400 scientists attended the Symposium and many of these participated in the discussions

One of the chief objectives of the Symposium was to provide an occasion for an exchange of ideas and information between workers in different areas of the virus and rickettsial fields The subjects discussed were grouped in five major categories Mechanisms of Virus and Rickettsial Infections which included twelve papers Ecology and Pathogenesis of Virus and Rickettsial Infections with six papers Mechanisms of Immunity in Virus and Rickettsial Infections with six papers Laboratory Diagnosis of Virus and Rickettsial Infections with five papers and Approaches to Prophylaxis and Therapy of Virus and Rickettsial Infections with six papers No attempt was made to draw lines between different varieties of viruses and rickettsiae Instead an effort was made to integrate information recently obtained in various fields concerned with this group of infectious agents Twenty of the papers were concerned mainly with studies on viruses infecting man or animals seven with viruses infecting bacteria three with viruses infecting plants and three with rickettsiae

After the Symposium the manuscripts of the papers and transcripts of

the discussions were returned to the participants for revision In the interest of prompt publication editing of the revised manuscripts and transcripts has been limited to the barest essentials and the participants have been spared the obligation of correcting proof

Information on virus and rickettsial infections has theoretical interest for scientists in a number of disciplines and importance for practicing physicians as well Hence a review of current knowledge in this field should have wide usefulness Furthermore the amount of new information which is emerging and the concepts which are now developing in the study of these infectious agents provide additional reasons for an appraisal of the findings and ideas of a large group of prominent workers The proceedings of this Symposium afford an opportunity for this

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Detroit Michigan
February 1954

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Moderator

Albert B Sabin

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The Hospital of The Rockefeller Institute
for Medical Research, New York, New York

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Cornell University Medical College
New York, New York

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Part I

Mechanisms of Virus and Rickettsial Infections

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John G. Kidd
Cornell University Medical College
New York, New York

1

The Initiation of Cellular Infection by Viruses

Alfred Gottschalk
The Walter and Eliza Hall Institute of Medical Research Melbourne Australia

It is probably true to say that viruses represent a manifestation of life at its lowest level of size when size is measured in units of mass However better than by their small size viruses as a group of living organisms are defined by their common feature of propagation in living cells only In order to multiply the virus has to bring its genetic material into effective contact with the metabolic apparatus of the host cell Probably those cells only are suitable host cells which can provide the chemical resources required for virus reproduction and in which the genetic units of the virus are able to gain control over the host cell s metabolic machinery diverting the enzymatic processes in the direction needed for the replication of virus genetic patterns and the eventual formation of a new generation of infective virus

In order to get access to the intricate structure of the cytoplasma harbouring the enzymic equipment of the host cell the virus has to make contact with the cell It would appear that two different mechanisms exist by which a virus host cell contact is established

(1) One group of viruses comprising vaccinia virus ectromelia psittacosis and others are able to multiply in a wide range of epithelial and other animal cells Their contact with the host cell may be visualized as a chance collision once in contact with the surface of the cell the virus particle is taken into the cell just like other foreign bodies (charcoal dyes etc) With these viruses there is no evidence for the presence at their surface of a mechanism ensuring close and selected contact with the host cell s surface

(2) A second group of viruses including the bacterial viruses the influenza mumps group and the Newcastle disease virus is restricted in its propagation to cells lining a surface in contact with the environment These viruses have evolved an elaborate effector fitting precisely a receptor at the surface of the host cell This effector receptor mechanism for attachment

of the virus at the surface of the host cell has been worked out over the past ten years in great detail for the influenza virus group and more recently also for the bacterial viruses as far as can be assessed the mechanism of virus host cell combination with influenza and bacterial viruses is closely related The attachment of the virus to the host cell is the initial step in viral infection and I will confine this paper mainly to the initiation of cellular infection by influenza virus for it is on this system that since 1945 the workers at the Hall Institute in Melbourne have studied the initial phases of virus infection

The first relevant observation was made by Hirst[1] when in 1941 he observed that influenza virus grown in the lining cells of the allantoic cavity of chick embryos agglutinates fowl guinea pig and human erythrocytes the virus particles act by being adsorbed to the surface of the red cells and forming bridges between the erythrocytes Virus haemagglutination is essentially an adsorption phenomenon Since the heat of adsorption not involving strong chemical bonds is usually small virus haemagglutination proceeds well at 0° C When Hirst allowed virus and red cells to interact at 37° C adsorption of the virus onto the red cells was followed by spontaneous elution of the virus into the medium That this phenomenon of adsorption and desorption involved more than a purely physical procedure was shown by the further observation that whereas the virus after its spontaneous elution from the cell surface is functionally intact the red cell is irreversibly changed so as to be no longer available for virus adsorption or agglutination Hirst clearly recognized the resemblance of the phenomena described to an enzyme substrate interaction the enzyme possessed by the virus forming as it were by its adsorption onto receptors at the red cell surface an enzyme substrate intermediate complex which after chemical interaction breaks down into regenerated enzyme and product i e into living virus and changed red cell surface

To obtain more direct evidence for this interpretation an approach by biological physical and chemical means was decided upon in our Institute

The first major biological contribution was the preparation and purification by Burnet and co workers[3] of a soluble enzyme from vibrio cholerae cultures This enzyme imitates nearly to the last detail the activity of the enzyme supposed to be present at the virus surface It stabilizes red cells in the same manner as does the virus making the cells unavailable for further agglutination The bacterial enzyme referred to as receptor destroying enzyme (RDE) has proved of the greatest value for the progress made in further research on the subject under discussion Burnet s finding strengthened considerably Hirst s suggestion of the enzymatic nature of the spontaneous elution of the virus from the erythrocytes

Another observation pointing in the same direction was the change in net electrical charge of the red cells after treatment with virus or RDE In 1948 Hanig[4] showed that the action of the PR8 strain of influenza virus on human erythrocytes resulted in a decrease in the electrophoretic mobility

of the cells as measured in phosphate buffer at pH 7 35 This investigation was extended by Ada and Stone[3] in our Institute to include various members of the influenza mumps Newcastle disease group of viruses Different strains were found to reduce the electrophoretic mobility value to different levels though none of them down to the level obtained with a purified preparation of RDE An indication of the extent of reduction is given in Figure 1

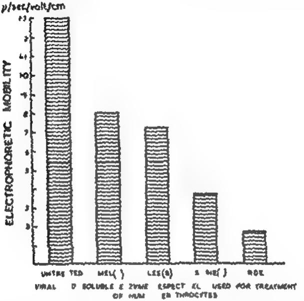

FIG 1

Further work showed that partial or complete pretreatment of cells with any virus strain followed by treatment with RDE reduced the electrophoretic mobility of the cells to the same value as that obtained with RDE alone The lack of any summation effect was taken as evidence that the same groups at the red cell surface which were susceptible to an attack by the various viruses were also sensitive to vibrio enzyme action The identification of the groups responsible for the decrease in electrical charge had to await chemical analysis

As mentioned already red cells pretreated with RDE do not adsorb virus The complete adsorption-elution phenomenon requires the unharmed living virus and intact receptors at the red cell surface It is possible however to alter by controlled damage either of the reactants in such a way as to allow adsorption but hinder spontaneous elution When for instance fowl or human red cells are treated with metaperiodate in the concentration range 0 2 to 2 0 mg periodate per ml of packed cells i e with M/100 to

M/1000 periodate the living virus is adsorbed to but not released from the periodated cells[6,7] The same over all effect is seen when intact red cells are mixed with virus previously heated to 55 C for 30 minutes This virus still adsorbed to the cells and agglutinating them has lost the capacity to change the receptors in such a way as to result in spontaneous elution of the virus[8]

These facts taken together left little doubt that the red cell surface harbours receptors serving as specific substrate for the viral enzyme and the closely related or identical enzyme produced by V cholerae However there could be some doubt whether it was permissible to apply the insight gained into the virus red cell relationship to the host cells of influenza virus since red cells do not support virus propagation It was therefore very gratifying to find that practically all the relevant phenomena observed with virus and red cells were reproducible when host cells were substituted for erythrocytes Again Hirst[9] was the first to demonstrate adsorption of the virus onto and spontaneous elution from susceptible respiratory cells of the excised ferret lung The same sequence of events was found in the excised mouse lung and Fazekas[10] has investigated this system extensively He found that active LEE virus is practically completely adsorbed to the lungs in 15 min and that at 20° C 50 min after administration spontaneous elution begins and that it comes to an end in 3 hours with about 75% recovery of the virus used RDE is able to interfere with this sequence in two different ways (1) When RDE is administered prior to virus installation the respiratory surface of excised mouse lungs is rendered incapable of adsorbing the virus (2) If

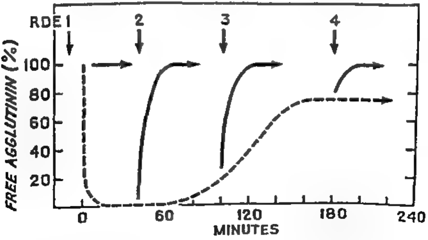

FIG 2 Action of RDE on adsorption and elution of virus in the excised mouse lung Dotted line represents the normal adsorption-elution curve of virus in untreated lungs Solid lines show the result of washing out the lungs with RDE at the time indicated by vertical arrows

administered at any time interval after virus addition RDE liberates in 5 to 10 min all residual virus from the lung cells Heat inactivated virus is adsorbed to the lung cells but without spontaneous elution following

The allantoic cavity of the chick embryo provides another easily available system containing cells susceptible to influenza virus infection Stone[11] has carefully studied this system with the following results

(1) If the allantoic cavity is washed with formalin thereby killing the embryo the virus is adsorbed to and eluted from the lining cells of the cavity in a fashion very similar to that described for red cells and the mouse lung

(2) When the formolized cavity is pretreated with periodate virus is adsorbed but not eluted

(3) Virus deposited in the allantoic cavity of living embryos is adsorbed by the susceptible cells this adsorption is followed not by elution but rather by infection of the cells

(4) The injection of RDE into the cavity of the living embryo prior to virus installation prevents infection for a period of 1 or 2 days when re generation of receptors restores susceptibility to the virus

It would appear that the accumulated data are conclusive evidence for the presence of distinct receptors at the surface of host cells These receptors provide the anchoring ground for the influenza virus and are therefore a prerequisite for virus infection The virus at its surface carries a specific enzyme which fits the receptor and can modify it

Obviously any further progress as to the nature of the cellular receptors and to the mode of the viral action had to come from chemical work Three observations paved the way for the chemical approach started in Melbourne in 1948 (1) Francis[3] discovered that influenza B virus upon heating to 55 for 30 min though not losing its capacity to agglutinate red cells was prevented from doing so by normal serum (2) Anderson[13] in our Institute showed that the inhibitory agent in serum the Francis inhibitor was a specific substrate for RDE and was rendered inactive by this enzyme and by the viruses of the mumps influenza group (3) Burnet[14] and co-workers[15] found that a great variety of mucinous material from human source (gastric mucin ovarian cyst mucin uterine cervical mucus saliva respiratory mucus) and from animals (sheep submaxillary glands egg white) contain a potent virus haemagglutinin inhibitor RDE or virus treatment inactivated the inhibitor

From the facts that the same specific enzyme irreversibly changes both the cellular receptors and the soluble mucins in such a way as to make them insensitive towards influenza virus and that the mucins are competitive in hibitors for the adsorption onto red cells of partially inactivated virus it was concluded that the cellular receptors and the mucins contained an identical or chemically related structure in other words we regarded the soluble inhibitory mucins as chemical analogues of the cellular receptors

M/1000 periodate the living virus is adsorbed to but not released from the periodated cells [6,7] The same over all effect is seen, when intact red cells are mixed with virus previously heated to 55° C for 30 minutes This virus, still adsorbed to the cells and agglutinating them has lost the capacity to change the receptors in such a way as to result in spontaneous elution of the virus [8]

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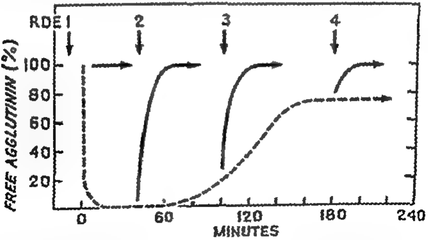

FIG 2 Action of RDE on adsorption and elution of virus in the excised mouse lung Dotted line represents the normal adsorption elution curve of virus in untreated lungs Solid lines show the result of washing out the lungs with RDE at the time indicated by vertical arrows

alysis might yield some information about the Ehrlich reacting component which was supposed to be present in the carbohydrate moiety By chromatographic and spectroscopical evidence we could identify glucosamine galactosamine galactose mannose and fucose as components [5] In addition the polysaccharide contained indeed the acid labile substance referred to above

Recently [6] we have identified this substance as pyrrole-2-carboxylic acid by comparing its properties with an authentic sample of the acid and

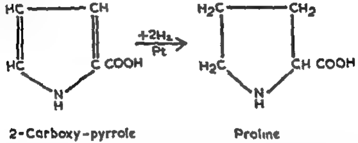

FIG 3

by its catalytic reduction to proline This imino acid as a pyrrole derivative gives immediately a strong Ehrlich reaction and when heated with dilute mineral acid in the presence of a reducing sugar gives rise within minutes to the formation of soluble and insoluble black humin just as observed with the split product We have found this imino-acid in all mucoproteins inhibitory or not and glycoproteins tested so far it is present in the compound released enzymatically from inhibitors and most probably it is a constituent of sialic acid a compound of unknown structure giving the Ehrlich reaction without any pretreatment first isolated by Blix[7] from bovine submaxillary glands

From the quantitative data available [8] a rough estimate may be made of the molecular composition of the carbohydrate complex of the urine mucoprotein

8 Hexosamine residues	
6 Galactose	mol weight of unit
3 Mannose	(3264)
1 Fucose	where $n < 5$
4 2-Carboxy-pyrrole residues	

The dializability through a cellophane membrane of the polysaccharide obtained from the mucoprotein by alkali treatment would suggest that n is not more than 3 or 4 We regard therefore the smallest unit (3264) as an individual prosthetic group rather than the repeating unit of a giant polysaccharide and visualize the mucoprotein as a conjugated protein con

We investigated first the interaction between purified influenza virus A (Melbourne strain) and ovomucin a protein fraction of egg white containing the virus haemagglutinin inhibitor It was found[16] that upon virus action a water soluble and dialyzable compound is released from the mucoprotein containing carbohydrate and a nitrogenous substance The compound had quite unusual features The carbohydrate was unstable against mild acid treatment at 100 for 10 min the nitrogenous substance or a component of it gave the Ehrlich reaction thought at that time to be specific for hexosamines however in contrast to the acid stability and alkali lability of the known hexosamines glucosamine and chondrosamine our hexosamine was acid labile and alkali stable Though at this stage the chemical analysis of the split product was unyielding its isolation was definite chemical proof for the enzymatic nature of the reaction studied Treatment of ovomucin with RDE resulted in the release of a compound very similar to that obtained with the viral enzyme No similar enzyme was detectable in the host cells of the virus or in the nutrient fluids of the chick embryo and it could be shown[17] that the enzyme is an integral and inseparable part of the virus particle itself

As mentioned before ovomucin is a protein fraction and with an adequate technique it was found[18] to contain not more than 5 to 10% inhibitor It was therefore of great interest and value to us that Tamm and Horsfall[19] succeeded in preparing from urine an inhibitory mucoprotein Quite independently Tamm and Horsfall [0] and Ada and Gottschalk [1] produced by a modification of the original method an electrophoretically homogeneous substance When highly purified influenza B virus (LEE) was allowed to act on the urine mucoprotein until its inhibitory faculty was lost a compound was separable from the reaction mixture by dialysis nearly identical with that obtained from ovomucin It resembled in many details amino acid N–glucosides or their isomers (N–substituted isoglucosamines) which meanwhile had been synthesized in our laboratory [3] In these synthetic compounds the carbohydrate is as unstable towards mild acid treatment as we had found it in the product released from mucoproteins by the viral enzyme The amino acid N–glucosides or their isomers differed however from the natural compound in that they did not give the Ehrlich reaction (without acetylacetone) These findings strongly suggested the presence in the inhibitory mucoproteins of a nitrogenous component other than hexosamine being *responsible for the Ehrlich reaction given by the split product*

Hirst[6] and Burnet and his group [4] had interpreted the modifying effect on cell receptor and inhibitory mucoproteins of low concentrations of periodate in terms of the well known oxidation of α–glycol groups in carbohydrates The presence in the split product of a sugar[15, 2] supported the view that the carbohydrate complex of the mucoprotein was subject to attack by the viral enzyme An analysis of the carbohydrate complex of the urine mucoprotein was therefore undertaken hoping that such an an

sults in adsorption of the virus to the host cell When both the polysaccharide and the virus enzyme are in their natural state adsorption is followed by enzyme action on the amide link of the pyrrole derivative If some α-glycol groups in the polysaccharide are oxidized by peroxidic acid adsorption of the virus to the cellular receptors still takes place though not followed by enzyme action For infection to take place only adsorption of the living virus to the host cell is necessary enzyme action on the prosthetic group of the receptors is not a prerequisite for cell infection as demonstrated by infection of host cells the receptors of which were modified by pretreatment with periodate [33] Once adsorption has taken place the virus is ingested into the cell an act proceeding at a higher rate than the spontaneous elution

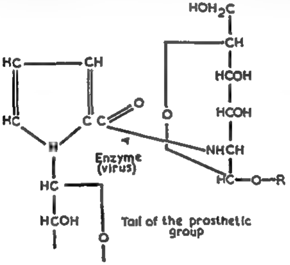

FIG 5

This then is the concept of initiation of cellular infection by influenza virus held at present by the Melbourne workers as the outcome of experimental work by many American virologists and by Burnet and his colleagues at the Walter and Eliza Hall Institute

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taining as *prosthetic* group a relatively small polysaccharide about two hundred of such polysaccharides may be linked to each protein molecule on the basis of a mol weight[20] of 7×10^6

It seems of considerable interest that the carbohydrate complex of the receptor substance present at the surface of human erythrocytes contains glucosamine galactosamine, galactose and fucose[29] and that the *prosthetic* group of the inhibitory mucoprotein prepared from human saliva[30] consists of hexosamine galactose fucose and 2–carboxy–pyrrole [31]

Concerning the linkage of 2–carboxy–pyrrole within the prosthetic group of the inhibitory mucoproteins it is suggested that its carbonyl carbon forms an amide link with the nitrogen of an adjacent hexosamine whereas the pyrrole nitrogen engages in a glycosidic linkage with a sugar residue Both such linkages would be alkali labile as they were indeed found to be [6] The pyrrole nitrogen has no basic property hence if the viral enzyme be an amidase its action on the amide link will add a positive charge to the residual mucoprotein not counterbalanced by an equivalent loss in the split product In fact it has been shown recently by Tamm *et al*[30] for the urine inhibitor and by Pye[3] in our Institute for all inhibitory mucoproteins prepared so far in a homogeneous state that the net negative charge sharply decreases after virus treatment or treatment with RDE just as had been found previously for the red cells [5]

These are the essential facts which have a bearing on the influenza virus host cell relationship under discussion We conclude from them that the first step in cellular infection by influenza virus is the adsorption of the infective particle onto the receptor substance at the surface of the host cell There can be little doubt that the cellular receptors are conjugated proteins that their *prosthetic groups distributed in pattern form over the protein* surface are heterogeneous polysaccharides and that the tail of these polysaccharides contains a pyrrole–carboxylic acid joined most probably by an amide link to a hexosamine residue and by an N–glucosidic link to a sugar residue At the surface of the influenza virus are areas with a pattern fitting the loose ends of the polysaccharides the chemical groupings building up these patterns are endowed with enzymic property Mutual attraction by electrostatic forces of the complementary areas at the influenza virus surface and at the free end of the prosthetic groups of the host cell receptors re

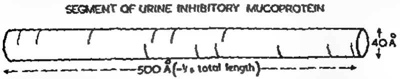

Fig 4 Showing a section of the thread like urine mucoprotein molecule (axial ratio of about 200) with the prosthetic groups in pattern form distributed over the surface

2

Some Central Problems of Viral Growth

A D Hershey
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It is generally accepted (Cold Spring Harbor Symposium 1953) that infection of bacteria by phage T2 starts off with the injection of the viral nucleic acid into the cell I shall not dwell on the evidence for this fact but pass immediately to what I consider its more interesting implications These may be stated in the form of four assumptions

1 Nucleic acid is the sole agent of genetic continuity during multiplication of the virus

2 All the nucleic acid of a particle of T2 is necessary for the production of a daughter particle but part of it should suffice when complemented by another particle for the production of a hybrid

3 Large specific pieces of nucleic acid are passed intact from mother to daughters during viral replication (or through concomitant recombination)

4 Viral protein has no function during viral replication insofar as this process can be distinguished from the maturation of infective particles

It must be insisted that the evidence for these assertions is incomplete for some of them it is negligible [2 4 7] The statements themselves would have been meaningless a few years ago and it is a measure of progress that each of them now suggests a number of lines of investigation that are perfectly feasible The program of research arrived at in this way has a special character however Practically all hope of learning something about growth and inheritance by it depends on the wager that some of the postulates to be tested are true

To examine a few consequences of these assumptions it is necessary to recall the scheme for which we are indebted principally to the work of Doermann [2] that summarizes genetic information about phage growth According to this scheme the infected cell contains noninfective particles called vegetative particles as well as infective particles The vegetative particles multiply and interact with one another to produce genetic recom

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binants The infective particles are terminal products of viral growth and are metabolically inert [5] The hypothetical vegetative particles are thus pre cursors of the infective virus

The genetic results call for a fairly large number of vegetative particles per cell Visconti and Delbruck[1] assumed at least 40 per bacterium and Levinthal and Visconti[8] arrived at the number 30 which is probably a minimal estimate

All this means that if the assumptions stated above are correct the in fected cell must contain nucleic acid that (a) is not contained in infective particles (b) has the purine and pyrimidine composition of the nucleic acid of infective particles (c) is a precursor of the infective particles and (d) measures at least 30 times the amount of nucleic acid present in one infective particle

Nucleic acid having all these characteristics has been identified in infected bacteria by Hershey Dixon and Chase [6] and by Hershey [5] Was this result to be expected on more general grounds? Evidently not if for example only a small part of the nucleic acid of the infective particle is contained in its germinal substance a possibility that had to be considered very seriously in the past [1 9 10 11] The analysis of precursor nucleic acid tends to show on the contrary that the vegetative phage particle contains approximately as much nucleic acid as the infective particle This can scarcely be called a confirmation of my assumption (2) but it is a step in the right direction

The assumption that viral protein has no function during viral replication suggests that the bulk of the protein should be formed somewhat later than the nucleic acid as part of the process of maturation of infective particles Some preliminary results of Maaløe and Symonds[10] are not inconsistent with this possibility The same may be said of the work of Luria and his collaborators referred to elsewhere in this Symposium However no attempt by purely biochemical methods has yet been made to fix the time of protein synthesis relative to other measureable features of phage growth Dr June Dixon Hudis is now beginning this attempt but the technical difficulties are likely to be considerable and a successful answer to the main question will be partly a matter of good luck

In conclusion I repeat that the problems I have discussed are far from solved and may remain so I have discussed them chiefly to air my opinion that the bacteriophages are worth a long close look with these problems in mind Fortunately some very capable investigators agree with this part at least of my discussion

References

1 Demars R S E Luria H Fisher and C Levinthal The production of incomplete bacteriophage particles by the action of proflavine and the properties of the incomplete particles *Ann Inst Pasteur* 84 113–128 (1953)

carbohydrate and phosphorus-containing moieties have been listed among its components [5] Studies in our laboratory have been undertaken to identify and count the groups responsible for the cellular surface charge These cells spontaneously agglutinate when the pH is lowered to about 4 5 a region where the ionization of the carboxyl groups is strongly depressed More over treatment with agents like propylene oxide or 95% $CH_3OH + 0\ 1$ M HCl which specifically block free carboxyl groups also neutralizes the surface charge to the point where the cells precipitate even at neutral pH It may be concluded that the negative surface charge of these cells is due in large part at least to the presence of free carboxyllate ions

The number of these negative sites has been estimated by measurement of the binding of three different types of positive radicals H^+ ion,[1] pos itively charged dyes like methylene blue and radioactive[3] $Zn^{++(65)}$ All three methods agree in fixing a value of approximately 3×10^7 negative groups on the cell surface a tremendously high value Accepting the maxi mal dimensions of these cylindrical cells to be 2μ in diameter and 4μ long a carboxyl group must be contained in each 100 square angstroms of the cell surface

The number of positively charged surface groups on cells of *E coli* B is as yet unmeasured but the following experiments show qualitatively that this number is much less than that of the negative sites

1 As noted in the absence of added salt cell suspensions begin to ag glutinate when the pH is lowered to 4 5 Further lowering of the pH even to zero does not re-disperse the cells If the positively charged groups were comparable in number to the negative carboxyls a point of re-dispersal of the aggregate would be expected within approximately one or at most two pH units below the initial precipitation point (Experiment has verified that all the changes described are reversible so that failure to re-disperse cannot be attributed to irreversible side reactions)

2 Treatment with formaldehyde which blocks ionization of amino groups has no measurable effect on the agglutination of these cells by Ca^{++} at various pH s (Fig 1)

3 Although these cells have a tremendously high affinity for positively charged polyelectrolytes they will not bind to molecules containing only negative groups at values of the ionic strength even in excess of that needed to produce virus host attachment [6 7]

4 Preliminary electrophoretic experiments indicate a high net negative charge on these cells at pH 7 [1]

These cells then are to be pictured as presenting a surface of extremely high negative charge density to the surrounding medium As a result of the electrostatic field thus engendered this surface must be in a condition of continuous strain due to the action of its unbalanced repulsive forces There will also be a strong tendency of this surface to bind positive ions from the medium or to hold them at a somewhat greater distance in an electrical

3

Cell Attachment and Penetration by Viruses*

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In this discussion the term attachment will refer to the initial reversible union between viruses and the outer wall of the host cell and the penetration process shall be considered to start with the irreversible reactions which follow thereafter and to end when the parts of the virus which function in synthesis have reached their target sites within the cell The penetration period is then the interval during which the cell is converted from a factory manufacturing cellular substance exclusively to one which in addition or instead synthesizes viral constituents At least some of the steps of the penetration process require a medium of only inorganic constituents so stored energy from virus or host must be utilized Understanding of the *nature of these reactions presupposes knowledge of the normal physical* and chemical state of cellular membranes as well as of the changes induced in them by virus invasion Actually the cell surface is one of the most obscure hinterlands of biology and the fragmentary data so far available afford no more than a suggestion concerning the nature of the processes initiated there by an attacking virus

Physico Chemical Structures of the Interacting Surfaces

The Host Cell Except where otherwise stated all experiments cited will deal with the $T2r^{+}$ bacteriophage and its host cell *E coli* B This strain of *cells like most bacteria possesses a strong negative surface charge as in*dicated by its electrophoretic migration[1] and its agglutination and precipitation when the pH of the solution is lowered The chemical structure of the cell wall substance has not yet been delineated although lipid protein

* Most of the experiments here discussed have been carried out as part of a program supported under research contract No AT (11 1) 269 with the Division of Biology and Medicine of the Atomic Energy Commission

precipitation of T2 begin at pH 4 8 and is maximal in the neighborhood of 3 0 indicates that here too the negative charge seems mainly to be due to ionization of the carboxyl group

The available evidence indicates however that the negative surface charge of the bacteriophage is appreciably less than that of the cell

1 The isoelectric point of T2 virus as judged by the point of minimum solubility is considerably higher than that of its host Thus T2 phage which begins to agglutinate at a pH of 4 8 precipitates maximally at a pH near 3 0 and begins to be redispersed when the pH drops below this value in contrast to cells which do not redisperse appreciably[8] even at pH of 0

2 In contrast to the cells T2 and other bacteriophages will attach to a negatively charged ion exchanger provided that a sufficient concentration of cations is present in the medium[7]

Phage T1 is of particular interest because a reversible electrostatically controlled rearrangement of the phage particle has been demonstrated[10,11] Hydrogen ion added to a suspension of T1 is bound to the virus thereby neutralizing a portion of its excess negative charge The resulting partially neutralized virus then undergoes a rearrangement which at 0 C is reversible producing a new form that we have designated as sensitized which still attaches to host cells but does not reproduce In the absence of cells the sensitized form of the phage can be restored to the normal form by raising the pH or by the addition of divalent cations which compete with H^+ ion for sites on the virus However a large excess of metallic cations can also produce reversible virus inactivation The rearrangement from the sensitized to the normal form is a relatively slow reaction with a half life of the order of 10 minutes in contrast to the actual ion binding and dissociation which appear to be practically instantaneous Although other viruses have not yet been studied in detail for this process there is at least some suggestion that similar effects may be involved Thus the shape of the pH inactivation curves of T5 and T7 at 0 C are identical and that of T3 similar to that of T1 where the sensitization process has been demonstrated to be responsible for the observed loss of invasive power Adams[12] has demonstrated a reversible dependence of plaque formation by T5 on Ca^{++} which cannot be satisfied by Mg^{++} or other cations The existence of different energy states of the virus which are triggered by electrostatic forces arising from ion binding may be important to an understanding of the subsequent splitting reaction

Anderson[13] has published electron micrographic studies indicating that T2 phage attaches to its host cell through its tail Recent immunologic studies[14] have demonstrated that the tail protein is structurally different from that of the phage head Finally it has been shown that neither T1 nor T2 bacteriophages can simultaneously attach to two or more host cells an indication that attachment sites are confined to a small area of the virus surface in contrast to the case with animal viruses like influenza[8] It may

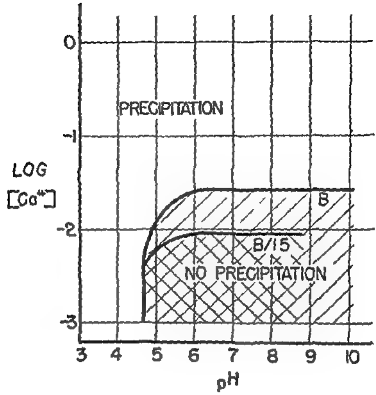

FIG 1 The Ca++- and H+-ion induced agglutination patterns of two cell mutants one of which is attacked by all the T system viruses while the other is resistant to attachment by T1 and T5

The curves indicate that the B/1 5 mutant has a lower negative surface charge than B which is a universal host The shapes of these two curves remain almost unchanged if the titration is carried out in the presence of a large excess of formaldehyde which blocks ionization of amino groups However if the carboxyl groups are blocked as by esterification the cells agglutinate spontaneously at pH 7 Hence the amino groups affecting the surface charge are much lower in number than the negative carboxyl groups

double layer The degree to which cations become bound to specific sites on this surface will alter its charge and probably its configuration as well As is to be expected these cells exhibit a tremendous binding affinity for cations like Zn^{++} less for Ca^{++} and Mg^{++} and still less for Na^{+}[5] Thus the reactions of such cells with virus particles or with other substances will be strongly conditioned by the charge relationships of the interacting bodies and the ionic constituents of the medium

Bacteriophage Structure The T system bacteriophages are composed of protein and DNA in roughly equal quantities these two constituents accounting for practically all of the mass[4] All of the viruses so far examined (T1 T2 T6) have negative charges at neutral pH's and the fact that

the metallic cations in promoting attachment lies more in their interaction with the virus than with the cell This conclusion is supported by two independent lines of evidence (a) The characteristic salt requirements for virus invasion are virus specific but not cell specific Thus T2 requires the same specific salt concentration for attachment to *E coli* B the universal host as to mutant cells like B 1 5 or B/4 despite the fact that such cell mutants differ markedly from each other in their surface chemical configurations [2] (b) The T system bacteriophages (and influenza virus) can attach to the negatively charged surface of a cationic exchange resin The attachment of each virus to such a resin displays the same specific salt dependence as does attachment to its host cell On the other hand the *E coli* host cells themselves do not become attached to a cationic exchanger even in salt concentrations far in excess of that required for cell attachment by any virus of the T system [7] It may be concluded that interaction with metallic cations changes the surface of the virus in such a way as to make possible its attachment either to the negatively charged ion-exchange resin or to a host cell

Since both components contain negatively charged sites those on the cell surface being functional while those on the virus interfere with attachment unless screened off by metallic cations it would be expected that in an excess of metallic cations ion binding to cellular carboxyls will become sufficiently extensive to depress attachment Such behavior is actually observed experimentally (Fig 2) The requirement for a greater concentration of Na^+ to accomplish the same effect as a given amount of Mg^{++} is doubtless due to the greater electrostatic attraction of the divalent ions (It seems likely that divalent cations function primarily through binding to the negative carboxyl groups and only secondarily through double layer formation whereas with monovalent cations the situation is reversed)

These considerations have provided an explanation for the interesting phenomenon that whereas T1 bacteriophage can utilize either monovalent or divalent cations for attachment to its host T2 appears to have a specific requirement for the monovalent ion [illegible] More recent experiments have shown that T2 can utilize Ca^{++} for host attachment but requires 0 1 M a disproportionately high amount compared to T1 However this much Ca^{++} binds so strongly to B as to cause strong cell agglutination which tends to mask the uptake of the virus

The bacteriophages display various types of ion specificities ranging from the simple dependence on the charge of the salt cations present exhibited by the attachment of T1 to its host cell (Fig 2) to a co-factor like action of specific cations which cannot be exercised even by closely related ionic species with the same total charge Examples of the latter behavior include the need for Ca^{++} but not Mg^{++} for host attachment of certain tryptophane requiring mutants of T4 [illegible] the specific action of Ca^{++} in promoting penetration of T5 [illegible] and the special action of Zn^{++} in blocking

be concluded then that molecular groups responsible for virus cell attach ment form a singular region on the virus surface and are presumably con fined to the tip of the tail

Nature of Primary Virus Cell Attachment The initial union of virus and host might conceivably occur through covalent bond formation as in establishment of S—S links between the two bodies or through electrostatic interaction involving ionic multipolar H bonding and Van der Waal forces Evidence for the electrostatic nature of the bond and for participation of the stronger ionic forces has been presented in previous papers [15 17] One of the salient features of the attachment is its requirement for inorganic cations Hence the possibility had to be considered that a divalent cation might form a bridge linking a negative charge on the surface of each body Such a divalent cation might be supplied directly from the medium or might be firmly bonded by one valence to one of the bodies with a remain ing positive valence free to grasp the other Such a picture has been proved untenable by experiments with ethylenediamine tetraacetate in which it was shown that concentrations of this chelating agent as high as 0 07 M, had no effect whatever on the attachment of T2 to its host cell[9] This amount of the chelating agent is sufficient to sequester divalent ions completely either from the medium or when attached by only one valence to any link age of the kind postulated

Study of the effect of various reagents on the attachment of T2 to B has yielded results which point strongly to invasive attachment requiring bond formation between positively charged amino (or substituted amino) groups on the virus and negative carboxyl groups on the cell surface [9 17] This evi dence may be summarized as follows (a) The attachment reaction is in hibited at pH s where either the carboxyl or amino group fails to ionize (b) Treatment of cells with reagents specifically blocking the carboxyl group destroys their ability to bind T2 while treatment with a variety of reagents which affect other groups like amino sulfhydryl etc has no effect on their affinity for T2 (c) Treatment of T2 phage with amino blocking reagents like acetic anhydride or a negatively charged molecule like a poly phosphate prevents its specific attachment to B while carboxyl or sulf hydryl blocking reagents have relatively little effect on this function

These data permit an understanding of the function of inorganic cations requirements for which have been established in every virus host attach ment system which has been carefully studied [8 1 18] For example T2 phage requires 10 20 times as high a concentration of NaCl as does T1 in order to attach to their common host In low ionic strength at neutral pH both virus and cell have so strong a negative surface charge as to prevent engage ment by the complementary bonding groups The presence of cations by bonding to and double layer formation around the negative groups of the virus permits its amino groups to approach and unite with the negative sites on the opposing surface This picture would demand that the importance of

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ical system highly discriminating If the system contains a number of different kinds of sites attracting the ion in question and if performance of the given biological function requires that only some of these chemical groups bind a cation but that other sites must be free then it is easily visualized how only one ion distribution function might be able to produce the particular electrostatic configuration among *all* the different charged groups involved which is required for performance of the function Thus in the virus cell system cations either by binding or double layer formation or both can (a) neutralize excess negative charge on the virus permitting its groups to engage complementary ones on the cell (b) neutralize still more virus negative groups and while permitting cell attachment promote a rearrangement of the virus to a form which cannot form a plaque (T1–sensitization) (c) neutralize negative groups on the cell and thus block some sites needed for attachment of certain viruses and (d) neutralize even more negative cell groups to the point where the cells agglutinate and settle out of the suspension Where cations can carry out so many different functions and where a careful balance between all the various possible degrees of neutralization of the various negative sites may be required for successful invasion of the host cell to be initiated even the simple ions may exhibit high degrees of specificity

Attachment of a virus to a cell even in the proper concentration of salt is not sufficient to insure penetration Thus the rate constant for attachment of T1 to the mutant cell B/1 is identical to that obtained with B at all temperatures and salt concentrations yet penetration of the former mutant never occurs[5,19] T1 which has been sensitized by a previous exposure to pH 4 can attach to B but will not penetrate even in optimal salt concentration as indicated by its failure to kill the host cell or reproduce therein T2 when treated with an excess of H^+ or with a synthetic polyamino electrolyte will attach both to B or the B/2 mutant but the succession of subsequent steps including cell killing virus splitting and multiplication does not occur[9] It may be concluded that penetration requires attachment in a specific manner between virus and host

It has been demonstrated that the basis of the specificity whereby mutant cells are differentially resistant to one or more viruses of the group for which the wild type is the universal host can lie in blocking of either the primary attachment or the penetration reaction[5] Both types of mutants have been isolated Evidence has been presented showing that the resistant mutants differ from the wild type in the number and distribution of carboxyl groups on their surface [8,16] In some cases a parallel difference in sensitivity to bactericidal poly amino compounds has been demonstrated a fact which suggests that the development of various drug resistant mutants may depend at least in part on genetically governed changes in configuration of surface groups which control binding or penetration of specific molecules from the medium [9]

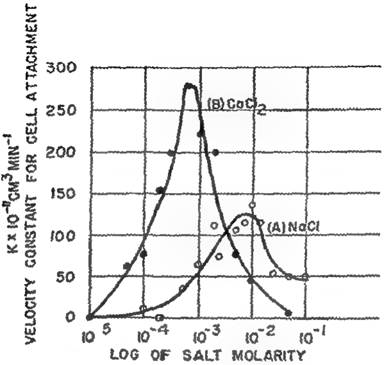


FIG 2 Effect of inorganic cations on the attachment of T1 to its host cell[15]

only the first step of T1 penetration which cannot be duplicated by Ca^{++} Mg^{++} or Ni^{++} [6]

The physico-chemical basis of the high degree of specificity often exhibited by simple inorganic ions in biological systems is difficult to understand It is usually explained in terms of a much higher binding energy between a specific ion and a critical site in the biological system While such unique differences in binding energy may often arise particularly in ions which display large amounts of coordination as well as electrostatic binding it is more difficult to visualize this as a sufficient mechanism in systems where members of the alkali or alkaline earth metals exhibit the specificity For if binding energy or double layer formation at a single critical site alone were involved it should be possible to find some concentration at which any of a large variety of cations would produce a similar electrostatic potential pattern about the key site The facts which have accumulated in connection with the bacteriophage system suggest a mechanism which may have general biological significance in explaining how small differences in binding energies may be amplified so as to make the biolog

or may simply reflect inherent instability of the virus with respect to dissociation T2 virus was treated with a large variety of reagents including acid (pH 1) alkali (pH 11) HNO_2 CH_2O and others at room temperatures and 37 C Despite the complete loss in virus activity which such treatment usually effected tests with differentially labelled radioactive phage showed no splitting of the protein and DNA components In view of this behavior the ability of a negatively charged ion-exchanger to split phage quantitatively at 0 within a matter of seconds would appear to bear a meaningful resemblance to the similar action occurring at the cell surface

Herriott[22] has shown that the killing function of the virus resides in the protein fraction alone He has also demonstrated that irreversible attachment may be separated from the cell killing step[23] a conclusion also reached by Watson from study of the behavior of X irradiated phage[24] A single virus particle can kill a cell apparently no matter where on the cell surface it happens first to engage Since the cross sectional area of the virus attachment site is approximately 1 50 000th or less of the cell surface it is difficult to understand how any kind of a change limited to so small an area placed almost at random on the cell surface could produce so profound an effect It would seem likely therefore that as a result of the attachment of a sufficiently large number of virus amino groups to negative cell sites a reaction is triggered which spreads over a distance much larger than that represented by the cross sectional area of the virus That the entire cell membrane is fundamentally changed as a result of virus attachment is revealed by the experiments of Graham[3] who showed that shortly after the successful infection of a cell by a virus the entire cell membrane becomes resistant to the injection of the DNA from a second virus It seems plausible to relate the ability of such a disturbance to spread over the cell surface to the strained electrostatic condition of the membrane

Lysis

If three or more T2 particles attach to a host cell in 0 02 M NaCl only reversible attachment ensues In 0 10 M NaCl the phages split and the cell is killed The sequence of these events seems to be the same at 0 C or at 37[8] The path of subsequent reactions however depends on the temperature and the presence of nutrients in the medium At 0 C little further change occurs At 37° C in the presence of adequate nutrients the penetration process is completed and new virus synthesis begins eventually leading to disintegration of the cell and discharge of the newly synthesized virus In the absence of nutrients (or even in their presence but with 40 fold more viruses attached to the cell) cellular lysis occurs without the synthesis of new phage (lysis from without)[25 7 8]

This abortive lytic phenomenon may have no relationship to phage growth as Watson concluded[4] it might be destructive triggering of the lytic mechanism which normally is called into operation only after the new

First Steps of Penetration Virus Splitting and Cell Killing

Hershey and Chase [1] first demonstrated that the DNA and protein portions of T2 virus become separated shortly after infection the former apparently penetrating the cell to take part in new synthetic reactions while the latter remains on the surface from which it can be removed without harm to the subsequent synthesis of new virus Our experiments have shown *that if T2 virus attacks susceptible cells in 0 02 M NaCl the attachment is* slow and completely reversible both for the cell and the virus [III] If the salt concentration is then raised to 0 10 M the virus loses its ability to detach in viable form and the cell is killed in the sense that it has permanently lost its capacity to reproduce Both of these changes occur within a matter of seconds [8] A possible explanation for these salt effects is as follows In 0 02 M NaCl a larger number of virus amino groups bind to the cell and irreversible sequellae ensue triggered on the cell by local neutralization of its high negative surface charge and on the virus by being brought into the strongly negative field at the cell surface

Study with radioactive labels of the splitting of the protein and DNA parts of the virus which takes place on the cell surface has yielded the following facts [8] (a) splitting is initiated within a matter of seconds after attachment and requires no organic molecules in the medium (b) virus splitting can occur at 0 C and proceeds readily on cells previously heated to temperatures varying from 60–85 C to inactivate cell enzymes In the latter case however most of the DNA is not injected into the cell but is liberated into the medium (c) with normal cells at 0 C and with a NaCl concentration of 0 075 M instead of 0 1 M complete splitting of the virus occurs at the cell surface but only a fraction of the virus DNA is injected the rest being released into the external medium Those cells in which the DNA failed to be injected remained viable (d) T2 virus can be split quantitatively at the surface of a synthetic ion-exchanger containing a high density of negatively charged groups This splitting resembles that occurring at the cell surface (which also may be regarded as a negatively charged ion exchanger) in its rate specificity of salt requirements and equal effectiveness at low and high temperatures [7]

We interpret these findings as follows Splitting of the virus into protein and DNA particles does not require functional cell enzymes but only attachment of a critical proportion of virus amino groups to a surface with an appropriate distribution of negative charges hence the separation of these two virus components appears to be triggered solely by the initial electrostatic stimulus of the bond formation Killing of the cell by the virus occurs as a result of a step which is prerequisite for injection of the DNA inside the cell

Control experiments[9] have been carried out to test whether the electrostatically triggered splitting of T2 into protein and DNA is indeed specific

electrolytes or T2 virus can be inhibited by a divalent cation like Mg^{++} when present in a concentration of 0 005 M or more

That this lytic action requires a fairly high density of amino groups on the polyelectrolyte was demonstrated by testing the action of molecules resembling the diethylaminoethyl acrylate polymer but in which varying proportions of the positively charged ethanolamine residues had been replaced by free carboxyl groups These compounds were obtained by copolymerizing diethylamino ethyl acrylate with free methacrylic acid in varying proportions Molecules in which the mole percent of the amino groups had dropped to 50% or less were completely without effect on *E coli* B even in concentrations a thousandfold greater than that at which the 100 mole per cent amino compound produces rapid lysis

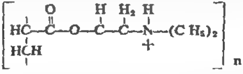
These experiments establish that attachment of a synthetic compound containing a large number of amino groups can trigger a lytic process in the cell which resembles that induced by T2 virus in requiring the action of a heat labile cellular component and in the kinetics of the reaction The high negative charge density at the cell surface with its consequent high energy state may be the reason why such reactions can be initiated by an electrostatic stimulus If this were the case one might expect that neutralization of some of the cell s negative groups by reagents which esterify carboxyl groups might also initiate the lytic reaction Such action is indeed observed Treatment of *E coli* B cells with propylene oxide or methanol plus HCl under proper conditions results in extensive cell lysis the kinetics of which resemble that produced by T2 virus or positive synthetic polyelectrolytes

Animal Viruses

All of the animal viruses which have been studied electrophoretically are negatively charged The kinetics of their cell attachment leaves little doubt as to the electrostatic nature of the reaction [illegible] Recent studies[80] have also revealed analogies between the irreversible reaction of influenza virus with the surface of the red cell and the phenomenon of lysis from without in bacteriophage both reactions occur in a medium containing only NaCl both are inhibited by low temperatures but not by ultra violet inactivation of the virus both require a multiplicity of several viruses per cell both result in loss of elements of the cell surface over an area far exceeding that of the attached virus particles Experiments with influenza virus have proved that virus attachment at the surface of the red cell initiates a spreading disturbance as has been shown to occur in *E coli* The two systems appear to differ in the following respects (a) Influenza virus itself appears to contain some enzymatic activity not yet demonstrated in bacteriophage (b) The destruction of red cell receptors by influenza virus releases the virus in active form in the medium whereas in T2 since the virus was split before lysis its activity is irreversibly lost and (c) The maximum number

phage synthesis is complete or it might be the reflection of a limited lytic mechanism which is part of the normal penetration process but which in the absence of necessary nutrients is not checked at the proper time and so destroys the cell [8] Such a mechanism might be imagined necessary to permit the huge DNA particle of the virus to be injected into the cell As Herriott first reported [2] lysis can be effected by the protein coats alone This fact implies that this lytic phenomenon is not an abortive triggering of the one normally occurring at the end of virus synthesis because that reaction still takes place even if the protein coat material of the initial infecting virus has been removed from the cell surface after penetration of the DNA Experiments performed in an effort to understand the role of this lytic reaction in the virus life cycle have established the following facts (a) Lysis from without does not occur in salt concentrations which permit only reversible virus attachment nor at 0° C in any salt concentrations (b) The virus protein and DNA are split apart when this lysis occurs as in ordinary invasion (c) Enzyme poisons like phenol and Hg^{++} prevent this reaction In an experiment to test whether such enzymatic activity is associated with the virus or the cells samples of T2 phage and B cells were separately heated to 60° C for 30 minutes Then the tubes were placed in a 37° C bath Normal cells were added to the heated virus and normal virus to the heated cells so that each tube contained the same final concentration of each component In both tubes rapid virus attachment occurred But whereas the tube with the heated virus lysed rapidly that with the heated cells showed no clearing of the suspension It may be concluded that this lytic reaction requires a heat labile cellular constituent [8]

Since lysis can be produced by the protein coat alone which attaches to the cell wall through its amino groups it was of interest to determine whether other amino-containing macromolecules could also elicit this reaction Such effects have been found with several types of molecules The behavior of the positively charged polyelectrolyte formed by condensation of diethylaminoethyl acrylate * and containing the repeating unit



may be summarized as follows [29] (a) Like T2 virus it will kill and lyse *E coli* cells about 5 10 times more material being required for the latter as the former Lysis occurs in concentrations of polyelectrolyte of 10 γ/cc (b) Lysis by the positive polyelectrolyte is rapid at 37° C but immeasurably slow at 0 C As with T2 lysis cannot occur under any conditions if the cells have been previously heated to 70° C (c) Lysis either by poly

* Kindly supplied by Dr W W Davis Physico-Chemical Division Eli Lilly Research Laboratories Eli Lilly and Co Indianapolis

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of sites on the red cell on which influenza virus particles can be accom modated amounts to only 2% of the total surface in contrast to the situation with bacteriophage where the virus appears able to occupy the entire sur face in close packed array[30]

Discussion

The experiments which have been presented lend themselves to synthesis in the following picture Attachment of the positively charged amino groups of the virus to carboxyl groups of the highly negatively charged cell surface electrostatically triggers a reaction in each body The virus splits into its protein and DNA components A migrating disturbance on the cell is al most simultaneously initiated which profoundly changes the entire outer wall and produced local lysis of the cell membrane which permits injection of the virus DNA

The following aspects of the penetration action still remain to be solved

a How does the DNA make its way from the interior of the virus to the cell surface?

b What is the mechanism of the virus splitting under the electrostatic stimulus of its attachment to a negatively charged surface?

c Does the DNA actually penetrate the cell through a hole lysed in the wall as a result of an enzymatic reaction triggered by the virus attach ment? The experiments cited dealing with the kinetics of the lytic action which is initiated by neutralization of cellular negative charges are only suggestive In this connection however Visconti s demon stration[31] that shortly after penetration a mechanism inhibitory to lysis from without develops in the infected cell is interesting Such a system could explain why the lytic reaction is self limiting in single infection (or in low multiple infection in the presence of adequate nutrients) but goes on to produce complete lysis otherwise

d What is the target site (or sites) inside the cell for the virus DNA?

The biggest single step forward in the study of the penetration process was the demonstration by Hershey and Chase that the bulk of the viral pro tein remains at the cell wall while the material penetrating seems to be only or mainly DNA The unanswered problems with respect to the penetration process are fundamental Yet it would seem that tools exist for obtaining at least some meaningful answers

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lowing infection even when further phage development is blocked by ultraviolet light or by lack of cofactors such as calcium and second the suppression of synthesis of specific polymerized host constituents such as enzymes and RNA We postulated a *parasitism at the genetic level* Phage genes would replace the incapacitated host genes and preside over the synthesis of phage specific polymers from unspecific materials manufactured by preexisting host enzymes

New evidence strengthens the concept of nuclear location and genetic role of the DNA of the infecting phage

1 In the lysogenic condition each cell carries latent phage in a "prophage" form which may occasionally give rise to mature phage spontaneously or by induction Infection leading to lysogenicity produces characteristic transient morphological changes in the host chromatin compatible with a process of nuclear penetration The prophage is apparently present in each lysogenic cell in single copy Following reinfection of lysogenic cells with mutant relatives of the latent phage there can be prophage substitution In the sexually analyzable K 12 group of *E coli* the lysogenicity for phage lambda segregates as a single factor with a specific chromosomal location This is presumably the prophage itself although segregation of a controlling host gene is not excluded by the published data

Maturation of the latent phage when it occurs appears to consist of a transition from prophage to vegetative phage followed by multiplication of the latter and maturation No phage antigens are found in lysogenic bacteria carrying the prophage

2 In *Salmonella* certain phages act as genetic carriers [13] Phage infection leading to lysogenicity (or superinfection of an already lysogenic and therefore immune bacterium) can rather infrequently transfer single traits of the strain on which the phage was last grown Here in the formation of phage particle the DNA becomes associated with apparently random portions of the host genome of which the phage particle becomes a carrier

3 In other cases the prophage itself or something regularly associated with it controls certain properties of the lysogenic cell Nontoxigenic strains of *Corynebacterium diphtheriae* are transformed into toxin producing strains by lysogenization with certain phages [14 15] the transformation occurs with a 100% efficiency

By analogy specific metabolic differences between normal and phage infected bacteria especially the synthesis of hydroxymethyl-cytosine for phages of the T2 group[16] and the synthesis of thymine induced by phage T2 in a thymine-deficient mutant of *E coli* [17] can best be explained in my opinion by a genetic role of the vegetative phage in directing the synthesis of the necessary enzymes Whether these should be called bacterial enzymes or phage enzym s is anybody s choice they are essentially enzymes of the phage infected cell

In summary the available evidence indicates close relations between

4

Genetic Functions and Developmental Processes of Bacterial Viruses*

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With most viruses infection of susceptible cells is followed by an eclipse period during which infectious virus cannot be recovered With bacteriophage this eclipse has been explained by Hershey and Chase s discovery[1] that the infecting virus particle separates into a DNA containing moiety which presides over new phage production and a protein-containing moiety which after functioning in adsorption appears to play no further role The development of new phage involves a series of processes synthesis of phage constituents multiplication of phage in vegetative form maturation of vegetative phage into infectious complete phage particles Other speakers in this symposium are dealing with the biochemical aspects of phage synthesis and with the multiplication of vegetative phage I wish to discuss briefly three problems that relate to the organizational aspects of phage production (1) the condition of the vegetative phage and of its relative the *prophage* in relation to cell organization (2) the production of phage proteins and their organization as immature phage materials (3) the relation between phage DNA and phage protein in the maturation process

The Condition of the Vegetative Phage and of the Prophage in the Host Cell

An intimate relation between vegetative phage and the genetic apparatus of the bacteria was first suggested on the basis of two groups of observations on virulent coli phages first the disruption of nuclear chromatin fol

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still without phosphorus These particles are present throughout the period of phage maturation and there is good evidence of their being actual phage precursors

In summary phage antigens in noninfectious form have been isolated as empty phage heads as ultrafiltrable materials related to phage tail and as apparently complete skins *but always without association with phage DNA* Yet there is no evidence that phage antigens ever arise except as a consequence of the multiplication of vegetative phage They are not formed in lysogenic bacteria In presence of proflavine when all the phage antigens are liberated in noninfectious DNA free form phage DNA nevertheless is synthesized in normal amounts [4] proflavine apparently prevents only the stable association of various phage constituents This brings us to our third and last problem

The Role of Vegetative Phage in Maturation

There is no knowledge of the biochemical mechanism by which nucleic acid may control protein formation The problem is two-fold the control of protein synthesis and that of protein specificity In line with other lines of work on protein biosynthesis phage data provide no evidence that the two problems can be separated

The isolation of DNA free antigenic phage precursors can be interpreted in two ways either the isolated materials are artifacts derived by disintegration of DNA-containing particles (in which case the ultimate step in maturation must be a sealing up of the particles) or the antigens of the phage skin are synthesized out of geometric context with the phage DNA

Even if the first hypothesis is correct there remains a serious question are the antigens of a given phage particle produced under the exclusive control of the DNA-containing core of that particle? This problem has been studied by mixed infection experiments

Unpublished work by Streisinger on phages T2 and T4 in our laboratory has shown that the heredity of the two recognized phage antigens is different In substrains derived from the progeny of crosses T2 × T4 the head antigens recognized by complement fixation with anti doughnut sera give a blending multigene heredity substrains produced by repeated crossings are intermediate in specificity between the two phage prototypes The tail antigens recognized by serum neutralization exhibit instead a single factor heredity The pattern of serum neutralization of each substrain is either that of one parent phage or that of the other The serum neutralization pattern is inseparable from the host range pattern A substrain serologically like T2 has the T2 host range and likewise for T4 Thus affinity for neutralizing antibody and adsorbability by specific hosts (both presumably tail properties) have common or closely linked single gene heredity

Phenotypically the situation as concerns tail properties is very different It was known that some of the particles emerging from a cross of T2 ×

phage in its reproductive forms (vegetative and prophage) and the hereditary apparatus of the host

The Production of Phage Specific Protein Materials

We come now to our second problem. Phage proteins are found in phage infected cells both in the new mature phage particles and as immature forms. With phages like T2 where infection stops production of many and possibly all nonphage materials [4,5,6] total protein synthesis has been variously reported to proceed[18] or to stop[19] following infection. Antigenic, morphological and isotopic studies of the production of phages T2 T4 and T5 have shown the following facts

1 During the first half of the latent period the antigens of the infecting particles can be detected in unchanged amounts by complement fixation tests on disrupted cells [6] These antigens are on the host surface and can be removed by the blendor technique of Hershey and Chase [1] In the second part of the latent period there is a formation of new nonremovable antigens which precedes by a few minutes the production of mature phage particles An excess of antigen over the amount accounted for by infectious particles is present throughout the rise period and is liberated upon lysis

2 Some of the excess antigens are present in morphologically characteristic particles found in prematurely disrupted bacteria [21] These particles or doughnuts resembling empty phage heads without tails have been purified from lysates of bacteria in which phage development is arrested by the action of proflavine The T2 doughnut is not adsorbed by bacteria it contains over 60% as much sulfur (protein) and less than 15% as much phosphorus (DNA) as mature phage It contains antigens that fix complement with antiphage serum but do not remove phage neutralizing antibody Serum against purified doughnut particles agglutinates phage particles headwise [7] Thus we identify a group of phage antigens as located in the phage head not involved in neutralization of phage infectivity by antiserum and appearing as organized particles a few minutes before the infectious phage No evidence of the presence of these antigens in nonorganized soluble form has been obtained

3 Another phage antigen that combines specifically with phage neutralizing antibody has been found in noninfectious form [2,4] It is present separate from doughnut particles in ultrafiltrates of premature or normal lysates of infected bacteria The ultrafiltrable material is not larger than 25–30 mμ a majority of this antigen however is not ultrafiltrable through phage retaining filters The ultrafiltrable fraction begins to be produced a few minutes before the infectious phage the production of the nonultrafiltrable fraction has not yet been investigated Evidence for a location of this antigen in the tail of the mature phage has been obtained [3]

4 In addition to separate head antigen and tail antigens immature phage particles have been recognized [5] containing both head and tail but

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T4 exhibit *phenotypic mixing* they have the T2 host range genotype but the T4 phenotype [6] In crosses T2 × T2h the progeny includes particles with T2 genotype and T2h phenotype and vice versa [7]

In crosses between partially isogenic substrains with T2 and T4 tail genotypes Streisinger finds that the progeny particles possess a variety of phenotypes Some particles have host range and sensitivity to neutralizing antibodies like T2 others like T4 others like both All categories include particles with the genotype of T2 and particles with the genotype of T4 The remarkable fact is that the tail phenotype of a progeny particle is not correlated with its genotype (beyond the extent accounted for by unequal numbers of parent particles in some bacteria) In addition the results suggest that the phenotypic mixing is not due to exchanges of parts among particles

The independence of phenotype and genotype and the existence of particles with the double phenotypes of the two parents taken together with the absence of double or intermediate genotypes and with the presence of ultrafiltrable tail antigens suggest very strongly the presence of a pool of tail materials The specificity of these materials would be determined by vegetative phage elements of either genotype and their incorporation into the maturing phage particles would take place more or less at random

In summary the circumstantial evidence favors a two-stage process in phage maturation with a synthesis of specific materials preceding a less specific assembly of the phage particle

One Virus, no Virus, or Many Viruses

Are some or all of the above features of phage production unique attributes of bacteriophages? Or are they shared by other viruses? Authoritative opinions are divided Rather than pointing out a long series of analogies it may be more useful to express a pragmatic utilitarian philosophy

No lead provided in the last 15 years by the study of any one group of viruses (be it growth cycle eclipse of infectivity developmental sequence serological organization or interference) has proved fruitless when followed with other viruses Extrapolation when used in experiment rather than in sheer speculation has been valuable both heuristically and interpretatively The very importance that would attach to a recognition in other viruses of any of the newly recognized properties of phage (relation with host genetic materials control over host properties latency and spontaneous or induced maturation) is in itself a valid justification for thorough exploration

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In the following I will describe first the conditions under which large yields of incomplete influenza virus are produced next the properties of this agent as compared with those of the fully active virus and finally some studies concerned with the mechanisms involved in the phenomenon

As has been mentioned the methods by which large yields of mainly incomplete virus can be obtained all involve the use of heavy inocula Following inoculation of undiluted Standard virus into Bernkopf s de embryonated eggs or into tissue culture[4] a considerable increase in hemagglutinin takes place without a concomitant production of infective virus In mice Ginsberg[10] found that non infectious hemagglutinin is produced in the lungs following inoculation of large doses of Standard virus provided that the strain employed caused lesions in the mouse lung As shown by Schlesinger[27] non infectious hemagglutinin develops also in mouse brains upon intracerebral injection of non neutrotropic strains of influenza virus In ordinary embryonated eggs inoculated with undiluted Standard" virus some incomplete virus is produced but there is also an increase in infective virus and the phenomenon thus appears less pronounced than for example in de-embryonated eggs However on continued transfer of undiluted seed the dissociation between infectivity and hemagglutinating activity increases and after a few transfers the yield contains only small amounts of infective virus although the content of hemagglutinin is high [1] With the PR8 strain such yields are obtained after 3–4 passages whereas with the Lee strain 5–6 successive transfers of undiluted seed are required

Studies of the properties of the incomplete virus have been carried out mostly with material obtained from eggs embryonated or de-embryonated[1] [2] The results of these studies have shown that the non infectious hemagglutinin as regards surface characteristics closely resembles the fully active virus Both forms are adsorbed onto and eluted from red cells in the same way They both have the same relative affinity for chicken and guinea pig cells and also the same stability to heat and to chemical influences The two forms can furthermore not be distinguished serologically they have the same antibody fixing capacities and the incomplete virus possess immunizing capacities—qualitative as well as quantitative—comparable to those of the fully active virus In the electron microscope finally both forms appear to have the same shape and size

However the incomplete virus differs from the fully active particles in at least three different ways (1) It is apparently non infective (2) It is less toxic[2] and (3) It is sedimented at a slower rate than is the fully active virus

Concerning the last point it has been shown that in PR8 Standard preparations the main virus component has a sedimentation constant of about 750 S whereas in preparations consisting mainly of incomplete virus the non infectious hemagglutinin is associated with somewhat hetero

5

"Incomplete" Forms of Influenza Virus

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When influenza viruses are grown in the laboratory according to the conventional methods i e when dilute inocula are employed as seed the relation between infectivity and hemagglutinating activity is remarkably constant When the hemagglutinin titer is measured by Salk s method [5] one hemagglutinating unit is found to equal about 10^6 egg infective doses[3]

An infectivity hemagglutinin ratio (I/A ratio) higher than 10^6 is never encountered In consequence it seems reasonable to conclude that the bulk of influenza virus particles present in a suspension with this I/A ratio is fully active i e endowed with both hemagglutinating and infecting activity This assumption is compatible also with direct countings of the number of particles constituting one infective dose and one hemagglutinin unit respectively [6]

For convenience passages carried out with dilute seeds will in the following be referred to as *Standard passages* and the yield obtained in such transfers will be termed *Standard virus* It will be assumed that in all instances where the infectivity hemagglutinin ratio is of an order of about 10^6 the main part of the influenza virus particles present are fully active i e infective and hemagglutinating

Under certain experimental conditions—which all involve the use of large seeds—virus particles are produced which are hemagglutinating but apparently non infective Preparations may be obtained which have an I/A ratio of only 1 100 or less indicating that out of 10 000 hemagglutinating particles only one is also infective The non infectious influenza hemagglutinin has been termed *incomplete* virus as opposed to the fully active complete infective virus

Similar incomplete forms have been described also in a number of other viruses but by far the most comprehensive studies have been carried out with influenza virus

Standard virus was incubated in vitro at 37 C At intervals samples of the fluid were removed and passed undiluted to eggs The virus growth curves induced by the partially inactivated Standard preparations clearly showed that heat inactivated virus plays at most only a minor role in the production of incomplete virus This conclusion was supported also by another experiment which clearly established that the factor responsible for the formation of incomplete virus develops early and parallel with the increase in hemagglutinin This experiment was carried out as follows A batch of eggs was inoculat d with undiluted PR8 Standard virus and groups of eggs were harvested at 1½ 3 and 6 hours after inoculation re spectively Both membrane suspensions and allantoic fluids from these eggs were used undiluted as seed for subcultures The 1½ hours harvest of membrane and allantoic fluid—both of which contained only small amounts of virus—gave rise to a production of fully active virus With the 3 hour membrane suspension—which contained larger amounts of newly formed "non infectious" hemagglutinin—the growth curve resembled that usually obtained in the second undiluted passage of PR8 virus i e there was only a limited increase in infectivity whereas the hemagglutinin titer reached high levels The 3 hour allantoic fluid seed—in which the hemagglutinin titer was still unchanged—gave rise to a Standard passage growth curve After 6 hours however the hemagglutinin titer of the allantoic fluid seed had increased and on subculture this seed induced formation of large quan tities of incomplete virus Samples of 1st passage fluid harvested 18 to 30 hours later on subculture gave rise to growth curves fairly identical to those obtained with the 6 hours sample

This experiment supported the conclusion that the formation of incom plete virus cannot be accounted for by heat inactivated seed virus and it indicated further that the atypical virus reproduction is due to the presence in the seed of certain amounts of non infectious hemagglutinin

The possibility has been suggested that the non infectious hemagglutinin might be a self reproducing virus variant which on continued transfer overgrows the normal virus However if this interpretation was true it should be possible by continued passage eventually to obtain pure prepa rations of this virus variant This has not been possible After 6 to 7 undi luted transfers of the Lee strain the allantoic fluids will contain predom inantly incomplete virus and on subculture these fluids do not induce any significant increase in virus infective or incomplete

These findings suggested that the formation of incomplete virus is de pendent upon the presence in the seed of certain amounts of infective virus and it seemed probable that the varying proportion of fully active to in complete virus in the seeds might account for the gradual development of incomplete virus as observed upon serial transfer of undiluted virus

In an attempt to test this assumption a qualitative analysis of the yields produced by more than 200 undiluted Lee seeds were analyzed in the

geneous material with a mean sedimentation constant of 500 S or less In such preparations no traces of the 750 S component was observed whereas in Standard virus preparations smaller quantities of the slower sediment ing component were occasionally observed indicating that Standard virus may contain certain amounts of incomplete virus [19] Since electron micrographs did not reveal any significant difference between incomplete and complete virus it may be concluded that the difference in sedimentation constants is due to a difference in density rather than to a difference in shape or size

In an approach to a better understanding of the origin and the nature of the incomplete virus it seemed desirable to study the mechanism involved in the atypical virus reproduction

As regards the formation of incomplete virus in de embryonated eggs and tissue culture the phenomenon appears to be dependent only upon the concentration of the seed Large yields of non infectious hemagglutinin are produced already in the first passage that is following inoculation of large doses of Standard virus Bernkopf considers it likely that the atypical virus production may be due to an overloading of the cells by virus It seems fairly easy to visualize that an overloading or saturation with virus may cause severe disturbances in the metabolism of the cells with the result that they are unable to support the complete intracellular growth of virus That cell damage may actually play a role in the formation of incomplete virus is indicated by the before mentioned observation by Ginsberg[10] that non infectious hemagglutinin is produced in mouse lungs only by strains which cause extensive lung lesions

In ordinary embryonated eggs however the gradual development of incomplete virus as observed upon serial transfer of undiluted seed shows that in this host the mechanism involved is more complicated in that the total content of virus in the seeds employed for the first 4 to 5 transfers of undiluted Lee virus is virtually identical for each passage There is on the other hand no doubt that in embryonated eggs too the concen tration of the seed is of major importance for the phenomenon Any seed which induce formation of incomplete virus will when passed in the diluted state give rise to formation of fully active virus It is however evi dent from the passage experiments that not only the *quantity* but also the *quality* of the seed must be of importance [23]

The possibility that the atypical virus formation was due to extra viral factors has been tested in various ways However attempts to separate the agents responsible for the formation of incomplete virus from the virus in the seed—by filtration high speed centrifugation or adsorption with red cells—all failed The available data all indicate that non viral factors do not play any role in the atypical virus production

Next the possible role of heat inactivated virus which might have accu mulated in the fluids employed as seed was studied Freshly harvested

On the basis of these findings it has been tentatively suggested that the formation of "incomplete" virus as observed in passage-experiments in embryonated eggs is to a large extent due to interference between fully active and "incomplete" virus. It seems possible that in cells infected with both fully active and "incomplete" virus the infective particles start to multiply and pass through the initial stages of the virus growth cycle but that the conversion of the immature particles into fully active virus is inhibited by the "incomplete" virus, possibly by annexation or blockade of some metabolic system of the host cell essential for this conversion.[13]

According to the working hypothesis just outlined the "incomplete" virus is supposed to act merely as an agent which inhibits the complete growth of the infective virus but which lacks completely the power of self replication. While this is undoubtedly true for "incomplete" 6th passage Lee virus, certain observations indicate that there may be qualitative differences between the "incomplete" forms derived from various undiluted transfers and that the "incomplete" virus produced in the first 4 to 5 undiluted Lee passages may actually possess some power of reproduction.

In the first place, careful studies of the virus growth curves at shortly spaced intervals have shown that in the 4th and 5th undiluted Lee passage the increase of virus occurs earlier than should be expected if the increase was due only to the infective virus in the seed. Secondly, the final yield obtained with 4th and 5th passage Lee seed were usually high and consistently contained 2 to 8 fold more virus than was obtained with artificial mixtures of "Standard" and 6th passage Lee virus although the infectivity and hemagglutinin content in these mixtures was identical to that of the "natural" seeds.

These findings would seem to indicate that the "incomplete" virus present in the earlier undiluted Lee passages has some ability to multiply, possibly by way of recombination processes similar to those described for bacterial viruses[14] and recently also for partially inactivated influenza virus.[15] In this connexion it is also of interest that Fulton and Isaacs[8] have recently reported that "incomplete" 3rd passage influenza virus is apparently able to multiply when grown in the ectodermal cells of the chorio-allantoic membrane. The interference between fully active and "incomplete" virus in the early undiluted passages may thus be comparable to interference between two active viruses, whereas in the 6th undiluted Lee passage the phenomenon is analogous to that observed between active and inactive virus.

Additional evidence that "incomplete" virus from various passages may differ qualitatively is finally supplied by sedimentation studies which indicate that the sedimentation constant of "incomplete" virus decreases with passage.

It will be realized that the available data concerning the formation of "incomplete" virus indicate that the mechanism involved is very complex and that probably several different factors add to the phenomenon.

following way In a diagram the number of infective doses and hemagglutinating units present in each inoculum was recorded along the vertical and horizontal axes respectively The composition of the resultant yield was recorded in the diagram

This analysis confirmed that yields consisting mainly of incomplete virus were obtained only following inoculation of large doses of virus i e more than 250 agglutinating units corresponding to $10^{9.4}$ virus particles[6]—or about 20 virus particles per cell [14]

This analysis showed also that when this requirement was fulfilled three different results were obtained according to the content of infective virus in the seed Seeds of high infectivity ($>10^{8.6}$ EI_{50}) induced formation of both infective and incomplete virus Seeds of medium infectivity produced mainly incomplete virus while finally seeds of low infectivity (less than 10^{6} EI_{50}) did not induce any detectable virus formation

The fact that seeds consisting predominantly of incomplete virus do not induce any virus formation is of particular interest As has been mentioned this observation is incompatible with the assumption that the incomplete virus is a self propagating virus variant It shows furthermore that the incomplete virus is able to interfere with and inhibit the propagation of the quite considerable amounts of infective particles present in the seed

Studies of the interfering capacity of the incomplete virus have shown that the interference may be at least partially overcome by large doses of Standard virus It was also established that several hours were required before the incomplete virus had rendered the susceptible cells completely resistant to super infection with fully active virus

These findings suggested that the formation of incomplete virus as observed in embryonated eggs might to some extent at least be due to interference between the fully active and the incomplete virus in the seed A direct approach to test this hypothesis was made by studying the virus growth induced by artificial mixtures of incomplete and fully active virus Serial ten fold dilutions of Standard virus were added to undiluted 6th passage Lee fluids which consisted mainly of incomplete virus Each mixture was passed undiluted to eggs and the growth curves were studied The mixture consisting of most infective virus ($10^{8.4}$ EI_{50}) induced formation of comparatively large amounts of incomplete plus smaller quantities of fully active virus The mixtures containing 10 and 100 fold less infective virus produced mainly incomplete virus and the total yield of virus was directly related to—although not proportional with—the concentration of infective virus in the seed The results obtained show clearly that the formation of incomplete virus is dependent upon the infective virus in the seed and they indicate that the incomplete virus in the presence of large amounts of infective virus suppress the formation of fully active virus rather than the formation of non infectious hemagglutinin

non infective plant virus particles also differ from the normal virus by their lack of nucleic acid

In recent years very similar non infective nucleic acid free incomplete forms of certain bacterial viruses have also been described [5 19 9] The incomplete bacteriophages have been shown to be present within the bacterial cell at a time when the virus has not yet been synthesized to the state of becoming infective and the available evidence that the incomplete phages represent immature forms of the infective particles seems rather convincing The striking similarity between the properties of the immature phages and those of incomplete influenza virus would seem to lend further support to the assumption that incomplete influenza virus too is an immature form of the fully active virus

As mentioned incomplete forms of bacteriophages and incomplete turnip yellow mosaic virus differ from the normal infective virus also in that they do not contain any nucleic acid So far only few attempts have been made to determine if there are similar differences between incomplete and complete forms of influenza virus and these attempts have not met with any success Such studies are too complicated for an ordinary virologist but it would be very desirable if a chemist with sufficient patience and energy would spend some time on this undoubtedly rather complicated and laborious job

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There is no doubt that multiple infection plays a dominant role for the production of incomplete virus However as has been mentioned small amounts of incomplete virus are present also in Standard preparations where as you will recall a very small inoculum is used The amount of incomplete virus produced in Standard passages seems to vary from strain to strain It seems probable that the formation of fully active virus is always accompanied by a production of *small* amounts of incomplete virus There is however no doubt that *large* yields of incomplete virus is produced only if the seed contains virus particles in sufficient numbers to cause multiple infection of the host cells

However multiple infection alone cannot account for the phenomenon as observed in embryonated eggs In these hosts the development seems to be due mainly to interference between the two forms of virus It seems possible that the formation of incomplete virus in de-embryonated eggs in tissue culture and in mice may also be due to interference In the two first mentioned hosts in particular it seems probable that the cells—because of the unnatural conditions under which they are maintained—may be deficient and that the comparatively small quantities of incomplete virus present in Standard virus inocula may be sufficient to cause a blockade similar to that seen in embryonated eggs only with seeds containing much larger amounts of incomplete virus

A similar interpretation may be applied also to the formation of non infectious hemagglutinin in mouse brains In mouse lungs on the other hand the available evidence would seem to indicate that in this host the phenomenon is more probably due to cell damage

As regards the nature of incomplete virus the various hypotheses concerning the mechanism responsible for the atypical virus formation all imply that the incomplete virus is an immature form of the fully active virus Conclusive evidence for this assumption has not been presented but it is at least to some extent supported by observations by the Henles[15] and by Hoyle[17] indicating that non infectious hemagglutinin appears in allantoic membranes prior to the appearance of infective virus

The formation of incomplete virus is as mentioned before not an exclusive peculiarity of influenza virus Similar forms have been observed in preparations of other hemagglutinating viruses as Newcastle Disease[11] and Fowl phage [9] Non infective virus material has been demonstrated also in certain plant viruses [1] The non infective particles observed by Markham and Smith [4] in sap from plants infected with turnip yellow mosaic virus are of particular interest because of the striking analogies between the properties of these forms and those of the incomplete forms of influenza virus The incomplete forms of both viruses resemble the normal infective particles in that they have the same surface characteristics as infective particles but they differ from the normal particles by being non infective and by having a lower sedimentation constant Chemically the

6

Studies on Double Infections With Influenza Virus

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The beautiful demonstration of genetic recombination between strains of bacteriophage by Hershey and Rotman[1] has stimulated the worker with animal viruses to search for the reproductive mechanisms that may be found in his own material Influenza virus has much to recommend it as an agent for genetic study and Burnet and his collaborators have already published a number of observations on experiments designed primarily to prove the existence of recombination in this group I shall confine my remarks however to interpretations of experiments carried out in my laboratory over the past two years experiments in which genetic recombination appears to play little if any role This work involves two new factors which will have to be dealt with in the future in considering the problem of influenza virus reproduction To attempt to give an overall picture today however would only involve us in wild and very probably fruitless speculation at a time when many new key experiments are possible but remain to be done

For two years Dr Tamar Gotlieb and I have been studying the results of simultaneous infection of the allantoic cavity with two influenza virus strains[3,4] We had hoped to find evidence of genetic recombination between our strains and for this purpose we decided to use virus antigens as genetic markers This proved to be a fortunate choice since a study of antigenic structure led us away from the problem of recombination and provided excellent evidence for the existence of two other factors involved in virus multiplication They may be described briefly as phenotypic mixing and heterozygosis Although it is quite certain that the two reactions are distinct a complete separation of their effects is difficult because (1)

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based will be discussed in a few minutes and at this point I will only point out that the occurrence of a few X_1 particles giving rise to *both* A and B could not be completely ruled out In other words particles of an A plus B phenotype were of genotype A or B This fits the description of phenotypic mixing and is similar to the bacteriophage model even to the extent that the antigens concerned in both cases have to do with the attachment of the virus to the host cell In spite of the fact that we do not have direct evidence that the distribution of agglutinating antigens in a host cell is a random one with respect to virus genotype nevertheless it appears to be a safe conclusion that the attachment of agglutinating antigen to a virus particle is a function which is independent of the genotype of that particle

The study of phenotypic mixing gives us some insight into the details of virus formation within the host cell although it is not primarily a genetic phenomenon In discussing it we drew our material largely from experiments with A and B virus since this permitted a study of the reaction in its purest form Most of our work however has been done with an A–A combination Melbourne and WSN (M and W) and we believe that it shows clearly the existence of another form of virus interaction heterozygosis

What we called X_1 virus was readily prepared by the simultaneous inoculation of M and W virus into eggs A number of experiments were carried out in an attempt to propagate the X form and as would be expected from the concept of phenotypic mixing these resulted only in yields of parent type viruses when the inocula were small On four or five occasions now the occurrence of an exceptional X form has been noted on such attempts at passage About one out of twelve of the first passage X_1 fluids induced the formation of a new type X virus when inoculated in small amounts into eggs This new kind of X virus was called X_2 and was distinguished from X_1 in its ability to be maintained by passage at high dilution Phenotypically X was like X_1 in many respects and it could be neutralized and the hemagglutinin inhibited by *both* parent sera It was also clear that X_2 gave rise to parent types M and W though apparently with a lower frequency than did X_1

X virus was carried on one occasion through more than twenty egg to egg passages utilizing at times material from eggs that had received but a fraction of one ID $_{50}$ of virus It seemed very probable that the line of descent passed through a single particle not once but several times and since both parent types (M and W) were present at the end of the passage series there existed a strong suggestion that a single particle of X had the potentiality of giving rise to both parent types Previously we had decided that a single X particle of the A–B variety could give rise to either A *or* B while now we wished to prove that an X particle of M–W type could yield both M *and* W Such a distinction promised to be diffi

they both may occur under the same experimental conditions and (2) because an accurate appraisal of the heterozygotic state is difficult by present methods

Phenotypic mixing is a phenomenon which occurs with bacteriophage[5] when two strains are used to infect the same bacillus and refers to virus particles which are genotypically like one parent but phenotypically like another Since very few studies have been published on this effect we are more than fortunate in having so much added evidence from Dr Luria this morning evidence which increases our understanding of the basic process very considerably

The pertinent facts can now be summarized briefly (1) Infection of a bacterial cell with T2 and T4 viruses is followed by a mixed yield of particles which are genotypically either T2 or T4 (2) The tails of virus particles produced are antigenically like T2 or T4 or they are of mixed character (3) The distribution of these three different types of tails is a completely random one among the particles of the two genotypes It is clear that the attachment of tails is a process which is functionally independent of the genetic material in the individual particles

The information on phenotypic mixing with influenza virus is not so detailed nor so complete as that with bacteriophage The clearest evidence comes from studies where one A and one B strain have been used for simultaneous inoculation The reason for this is that the second reaction heterozygosis is not a prominent feature of A–B infections and hence the picture is less confused A and B viruses are antigenically completely distinct from each other and an antiserum against one strain is without effect on the other in either hemagglutination inhibition or neutralization tests However when large and equal (in terms of ID$_{50}$) amounts of one A and one B virus were inoculated into the allantoic sac the resulting yield often consisted predominantly of virus in which both A and B antigens occurred on the surface of single particles The hemagglutinin of such doubly antigenic virus was inhibited and the virus was often neutralized by *both* A and B antisera

Virus of this mixed antigenic character we have called X_1 and we have succeeded in producing it with three different pairs of strains in which one virus was A and the other B several pairs where both strains were A and with one combination of influenza A and Newcastle disease virus[6] By proper manipulation of the proportions of two infecting strains it was possible in some cases to obtain yields of virus in which 95% of the particles were X_1 in type Fraser has also described what was undoubtedly phenotypic mixing following a double infection with two A strains

Recent experiments with X_1 virus of the A–B type have led to the conclusion that such particles are genotypically either A or B but probably not both The nature of the experiment on which this statement is

ploid product able to reproduce only slowly and able to compete with haploid virus only by virtue of the fact that *all* cells of the allantoic sac are infected in the beginning At least our heterozygotes and incomplete" viruses are best produced under the same conditions namely very large inocula

Only brief mention will be made at this time of the type of virus stocks isolated from infections initiated by X particles (M–W type) As stated before the major components of X breakdown are parent type strains and a stable form (X_3) which is most like the W parent[4] X_3 differs from W in that it has a distinct M antigenic component which can be detected by neutralization tests A second and very striking difference is that X_3 [redacted] not virulent for mice when given by the intracerebral route Reciprocal effects have not been found in M virus from X lines While these changes may be the result of *recombination of genetic characters* in which some of the segregants are lost a much more intriguing possibility [redacted] that the *shift from X_1 or X_2 to X_3 (diploid to haploid state)* [redacted] accompanied by an unequal distribution of the total genetic material to the two progeny and that this leaves X_3 heterozygous for part of the specific antigenic complex A close analogy for this picture comes from the breakdown of diploid forms of [redacted] coli found by Lederberg *et al*[9]

As I stated at the outset I do not intend to offer any general overall theory to cover the observations made by others and by ourselves on influenza virus multiplication No benefit will be derived from synthesizing a picture of the *process of replication* by taking bits of influenza research and interlarding them heavily with conceptions taken bodily from the study of bacteriophage behavior The academic and the practical consequences of understanding the reproductive mechanisms of animal viruses will be very great when achieved but our knowledge of this field is so primitive today that what I have emphasized this morning may best be described as mere starting points for further work

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cult, especially since we had only the limiting dilution method for the isolation of descendants of single particles

In order to use the limiting dilution technique for this purpose it was necessary to study virus infectivity endpoints in a quantitative manner. This type of study had already been done by Lin and Henle[4] with mixed infections of A and B. Our control studies with the same and other strains confirmed the essential features which they found. Two viruses were mixed *in vitro* in a ratio of one to one. The mixture was diluted in twofold steps and each dilution inoculated into 40 eggs. The range from 50% to zero infectivity was covered. After a suitable incubation period, each egg was examined to determine whether it had supported virus growth and if so whether one or two types. In control experiments it was found that the number of double infections which occurred was a fairly reproducible function of the number of ID_{50} of virus inoculated, regardless of the strains employed.

X_1 virus from A–B combinations was tested for the yield of doubly infected eggs at and beyond the point of 50% infectivity and no sharp difference from the controls was found, i.e. the number of eggs yielding both viruses could be explained by the chance inclusion of both genotypes in the inoculum. In marked contrast to this result, the inoculation of X_1 and X_2 virus from M–W combinations at high dilutions yielded a very high percentage of doubly infected eggs compared to the controls. The results were consistent in a number of tests and the differences were well beyond the limits of error of the methods

One explanation of the high incidence of double infection could be aggregation of the infecting particles. However, experiments on the sensitivity to ultraviolet light of X and the reversion of X to X strongly suggest that the explanation is a genetic one. From this standpoint, we may say that some particles of X_1 and X_2 are heterozygotes in respect to antigenic type. It is quite possible and even likely that, when more characters have been examined, it will be found that the X forms are heterozygous for many traits and may, in fact, be diploids. The heterozygotic state seems to be a relatively unstable one with frequent reversion to the original forms

There is little doubt in my mind that the heterozygotic or diploid state will be found to be an important one in the process of influenza virus reproduction. If genetic recombination occurs, then diploidy may well precede the exchange of characters. The occurrence of M–W diploids suggests that M–M and W–W types may occur as well and, while the latter would serologically be like the parent types, there might be other ways of detecting this condition. It is conceivable, although only a wild guess at the moment, that different degrees of polyploidy might be associated with varying degrees of viability and that the apparently non-viable hemagglutinin seen after very large inocula may be some sort of poly-

beautiful work of Dr Lwoff and his colleagues on the exciting effects that can be produced by various ionic unbalances in preventing or producing induction phenomena in the lysogenic viruses Moreover only 02 M sodium chloride is required for the attachment of T2 to its host but the subsequent steps of its metabolism require a five fold greater amount of this salt I think it would be safe to assume that most of the metabolic reactions involve some kind of concomitant ionic participation

With respect to the next question T1 virus which has been sensitized by a temporary exposure to low hydrogen ion concentration attaches readily to host cells but the cell is not killed Presumably then the DNA does not penetrate under these conditions

DR. SABIN I was waiting for the experts to comment but now perhaps there is time for general questions I am sure that the titles are not misleading anyone namely that we are not dealing here with penetration by all viruses but with penetration of the bacterial viruses under study I am sure that everyone is aware of this The question that I would like to ask is this Has it been shown as yet that any virus that does not possess a tail infects a bacterium by injecting or leaving in the cell only a portion of its total substance e g DNA? That is question #1 Secondly if we take a relatively large model namely the fertilization of the ovum by the spermatozoon the tail somehow or other gets left outside and the rest of the spermatozoon gets into the ovum and begins its work there Perhaps a large virus like vaccinia may function in that manner There may be many mechanisms by which viruses may infect cells Some of us now are inclined to say "Oh well viruses don t get inside the cells that s an old fashioned idea it s been shown that they only inject a little bit of their stuff into it " I would like to get a little bit of comment on that.

Now about the reaction of viruses with red cells While it is quite true that the influenza viruses and perhaps others work that way on the red cells I think the viruses affecting the nervous system which also hemagglutinate red cells react in quite a different fashion This is no criticism of the work presented here this morning but perhaps we can get some comment from the people on the program

DR HERSHEY I think Dr Sabin s point that we should not generalize prematurely about viruses is a good one I would go one step further than he does and suggest that the tailed viruses that is the larger bacteriophages represent specific adaptations to cells with sturdy walls Following this line of thought one sees no reason to expect that animal viruses generally will be found to infect cells by an injection mechanism. However now that we know this mechanism exists it is only a matter of hard work to find out which viruses use it

DISCUSSION

Mechanisms of Virus and Rickettsial Infections

DR LURIA Has it been established that phage T1 when attached to the cell without further growth does not introduce its desoxyribonucleic acid into the cell? Also ions may play roles in some of the early reactions following phage attachment Approximately one half of all bacteriophages require calcium for reproduction after attachment and we have found that calcium is specifically required for the penetration of DNA into the bacteria

DR JOHN W REBUCK (Henry Ford Hospital) I would like to ask Dr Gottschalk how his analyses of the receptors can be compared with current analyses of the blood group substances I would also like to ask him if he would discuss the findings of Abramson on the failure to change electrophoretic mobility of sensitized erythrocytes that is those sensitized by their specific agglutinins in the light of the electrophoretic mobility changes that he discussed

DR GOTTSCHALK With regard to the blood group substances which have been so exhaustively investigated by Morgan one can now say that though there are common features between the carbohydrate group of the blood group substances and the carbohydrate of mucoprotein both of them containing hesoxamine galactose and fucose they are definitely not identical Highly purified blood group substances have practically no virus antihaemagglutinin qualities and the influenza virus receptor substance from human red cells have very little blood group activity I think the difference lies first in the sequence of the individual sugars and in the manner in which they are linked together and secondly in the fact that virus haemagglutinin inhibitory mucoproteins have a much higher protein content than the blood group substances which are predominantly polysaccharides With regard to the second question the change in mobility of the erythrocytes following virus action is due to the unmasking of a charged group resulting from the splitting of a chemical bond In the case of the sensitized erythrocytes the adsorption of antibody and complement to the cell surface presumably does not affect the total net charge of the red cells

DR PUCK In answer to Dr Luria s question about the role of ions after completion of the initial attachment I am very sorry if by emphasizing the function of ions in the early steps of virus invasion I may have given the impression that I think they have no role later on This is far from the case In addition to those examples Dr Luria cited there is the very

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some observations that are pertinent to that Bacterial cells appear to have attachment sites spread over their complete surface so that the number of viruses which can attach to the single cell is equal to the number which you could fit on there in a close packed array something like a thousand viruses for example in the case of T1 In the case of the host cells or viruses like Newcastle s disease however these cells although they are much larger than the bacterial cells have many fewer attachment sites As a matter of fact the total number of attachment sites appears to be of the order of magnitude of a hundred despite the fact that these cells are thousands of times larger If as it seems to us to be worthy of consideration these attachment sites also contain the penetration cell sites through which the virus can initiate secondary injury to the cell one might then expect that in animal cells the ability of the cell to recover from such an engagement with a virus would be much greater

DR GEORGE ■ GEY (Johns Hopkins University School of Medicine) In working with eastern equine encephalitis virus grown in certain cell strains and with polio viruses grown on human tumor strains A Fi and HeLa minute peripheral portions of cells can be demonstrated to be destroyed when the remainder of the cell is still intact This has been observed in living cell preparations indicating that if one could imagine interference at the proper time the cell could recover from such infestation The point has been brought up about recovery and the possibility that a whole cell once exposed might recover as the result of exposure to virus We have actually taken motion pictures which demonstrate that portions of cells can be destroyed by polio virus before the whole cell succumbs Also in work with Dr Bang s group we have gotten electronmicrographs showing the effect of eastern equine encephalitis virus which tend to support this interpretation of a progressive destruction of cells Other modes of destruction of animal cells no doubt also occur

DR HORSFALL I would like to ask Dr von Magnus to explain what seems to be a discrepancy As I viewed the results it appeared that ■ the total quantity or the ratio of noninfective virus increased during serial passages the yield of noninfective virus in the next passage also increased Then at a certain point ■ g with PR8 between the fourth and sixth passage this correlation breaks down and suddenly the capacity to produce infective virus and relatively little noninfective is regained The same situation holds as passages are continued and more and more noninfective particles appear as more and more are inoculated until finally the correlation again breaks and we return to a relatively infective yield Is there an explanation for this seeming paradox?

VOICE I would like to add the following to what has gone before First of all I think everyone is fully prepared for the fact that even some phages might be found which inject part of their protein along with their DNA The most interesting aspect is to find a virus whose reproductive apparatus is contained in the smallest possible component and this is I think the reason why everyone has been treating Dr Hershey s discoveries with such interest and respect But I am sure that no one intends to read into this a general law to exclude other things The second thing is that if when obvious differences between viruses like influenza and some other mammalian viruses from the bacteriophages is that at least for the red cells they have several attachment sites as can be demonstrated by the phenomenon of agglutination Both the red cells and their host cells None of the bacterial viruses that we have ever studied under any conditions will agglutinate their host cells indicating that they have only one attachment site It is conceivable then that other viruses having only one single attachment penetration site could have such sites spread over them just as the host cell has portals of entry spread over a large part of its surface

MR BAWDEN Dr Hershey has said that no viruses are known that attack organisms like yeast which have tough cell walls This is not quite correct for flowering plants with which I work have exceedingly tough skins but they suffer from plenty of virus diseases However I am not questioning his general thesis for all uninjured plants seem to be immune from viruses which can enter only through wounds Bacteriophages differ most strikingly from viruses of flowering plants in their remarkable ability to pass unaided from one organism to another A virus in one flowering plant stays there unless someone or something takes it out and puts it into another In nature the something is usually a particular insect and it has long seemed to me that bacteriophages behave much more like plant viruses plus their vectors than the plant viruses alone Dr Hershey s fascinating results now suggest that the protein coat of the phage particles may indeed play the role of a vector This nicely explains one difference between the behaviour of phages and plant viruses but unfortunately it raises another where previously the two kinds of virus seemed to be reasonably similar If only the nucleic acid from phages enters bacteria chemically the phages seem to come more in line with transforming factors than with the plant viruses for these are nucleoproteins and in many of them the nucleic acid is quantitatively only a very minor component

VOICE If I may I would like to say something about that point because Doctor Levine and his associates in our laboratory have made

some observations that are pertinent to that Bacterial cells appear to have attachment sites spread over their complete surface so that the number of viruses which can attach to the single cell is equal to the number which you could fit on there in a close packed array something like a thousand viruses for example in the case of T1 In the case of the host cells or viruses like Newcastle s disease however these cells although they are much larger than the bacterial cells have many fewer attachment sites As a matter of fact the total number of attachment sites appears to be of the order of magnitude of a hundred despite the fact that these cells are thousands of times larger If as it seems to us to be worthy of consideration these attachment sites also contain the penetration cell sites through which the virus can initiate secondary injury to the cell one might then expect that in animal cells the ability of the cell to recover from such an engagement with a virus would be much greater

DR GEORGE O GEY (Johns Hopkins University School of Medicine) In working with eastern equine encephalitis virus grown in certain cell strains and with polio viruses grown on human tumor strains A Fi and HeLa minute peripheral portions of cells can be demonstrated to be destroyed when the remainder of the cell is still intact. This has been observed in living cell preparations indicating that if one could imagine interference at the proper time the cell could recover from such infestation The point has been brought up about recovery and the possibility that a whole cell once exposed might recover as the result of exposure to virus We have actually taken motion pictures which demonstrate that portions of cells can be destroyed by polio virus before the whole cell succumbs Also in work with Dr Bang s group we have gotten electronmicrographs showing the effect of eastern equine encephalitis virus which tend to support this interpretation of a progressive destruction of cells Other modes of destruction of animal cells no doubt also occur

DR HORSFALL I would like to ask Dr von Magnus to explain what seems to be a discrepancy As I viewed the results it appeared that as the total quantity or the ratio of noninfective virus increased during serial passages the yield of noninfective virus in the next passage also increased Then at a certain point e g with PR8 between the fourth and sixth passage this correlation breaks down and suddenly the capacity to produce infective virus and relatively little noninfective is regained The same situation holds as passages are continued and more and more noninfective particles appear as more and more are inoculated until finally the correlation again breaks and we return to a relatively infective yield Is there an explanation for this seeming paradox?

DR RIVERS (Moderator) Dr Horsfall may I ask you a question? Do you mean to say Is this virus coming or going? Is it a growing virus that is not mature or is it old virus that is breaking down?

DR HORSFALL I was very careful Dr Rivers not to use the words that you used or the words that Dr von Magnus used I referred only to non infective and infective virus particles but did not use any of the other words which I think have odd connotations

DR RIVERS Dr von Magnus do you want to answer that question? Not mine but Dr Horsfall s?

DR PREBEN VON MAGNUS I think the explanation of that is that when you get onto the fifth or sixth passage you get almost no virus production so in the sixth passage there is actually no increase of virus and when you harvest this fluid say at 18 hours after you have seeded the eggs this fluid contains about ten to fifty fold less virus than other previous seedings did So when you proceeded to new eggs the total content of virus in the 6th passage seed is too small to cause multiple infection of the cells You will get a part of the cells of course infected with incomplete virus while the remaining cells remain susceptible are not blocked so the active virus which is actually present in the seed would be able to multiply and to reach very high titers Now when you harvest that passage i e the 7th passage 18 hours after it has been inoculated then you have got a fluid in which there is a fairly high infectivity titer but in which still no measurable amount of incomplete virus is produced so you are back to almost a standard passage or to an in between standard passage and first passage fluid In this way you can repeat the cycle the increase in incomplete virus will gradually increase as through the first four passages then follows a passage with no increase in virus then an increase of infective virus and for some further passages you get mainly complete virus produced when you harvest the first 18 hours after inoculation Probably you would get quite different results if you harvested later on Then later on something more curious happens which I am not able to explain and which I have been careful to avoid today

DR LURIA In connection with the incomplete or non infectious particles of influenza a recent paper by Cairns suggests that there may be no such thing as non infectious hemagglutinin in the allantoic membrane According to Cairns treatment of de-embryonated infected eggs would immediately liberate all the hemagglutinin in the form of free infectious virus His conclusion is that the hemagglutinin is only formed as the virus comes out of the cell but the virus remains stuck on the surface of the cell for about an hour

DR PREBEN VON MAGNUS I think the experiments by Cairns are not directly comparable with those in embryonic eggs which are not inoculated with RDE repeatedly I would think that he has got results that are somewhere intermediary between what you get in de-embryonic eggs and what you get in ordinary embryonic eggs; At least we could not repeat his experiments in embryonic eggs

DR HAROLD S GINSBERG (Western Reserve University School of Medicine) I would like to try to compare briefly some of the findings of von Magnus in the chick embryo and those obtained in the mouse lung It is very difficult actually to be sure whether or not we are really dealing with the same phenomenon There are some very marked differences that arise The most essential differences I think are (1) non infectious virus arose on first passage a fact which he pointed out to occur in the de-embryonated egg and in tissue culture as well and (2) it was impossible to pass non infectious virus in the mouse lung On first passage of lung suspension which had a very high titer of non infectious and low titer of infectious virus there emerged fully infectious virus again and nothing that could be termed non infectious virus In addition if one employed short intervals after infection to examine the lungs in order to observe the initial cycle of multiplication it turned out that a very peculiar phenomenon arose and this was very reproducible approximately 3½ hours after viral inoculation non infectious virus could first be detected and there was a simultaneous decrease in quantity of infectious virus It appeared almost as though infectious virus were being converted into the non infectious form Experiments were then designed to compare the actual total amount of virus that is antigenic viral material in mouse lungs which contained a high titer of non infectious material with mouse lungs which contained chiefly infectious virus These experiments indicated that there was approximately the same quantity of viral material in each From experiments carried out in the mouse lung it is impossible to determine whether the non infectious virus formed is immature or incomplete virus except incomplete in the fact that it is non infectious Indeed it is very difficult to be certain that this viral component is not derived from infectious virus which when synthesized was rapidly converted into a non infectious form by some mechanism perhaps connected with cell damage

DR GOTTSCHALK Dr Burnet has asked me to make the following contribution to this discussion on recombination

We have been studying the phenomenon of double neutralization of hemagglutinin from mixed infections for the past two years Reference to it is made in Frazer s (1953) paper and another is in the press The phenomenon is well shown in de-embryonated egg experiments using our standard M+/WS− mixture Under these circumstances the infectivity of

the first cycle fluid is low and the great bulk of the doubly neutralizable hemagglutinin must represent incomplete virus Passage in first cycle de embryonated egg experiments is therefore only possible for two or at most three generations We have never obtained a true breeding clone of virus showing the doubly neutralizable character and are inclined to regard the phenomenon as quite different from true recombination

Our current hypothesis is that all or most influenza virus particles carry *more than one complete genome and that where these differ, the progeny* derived from an infected cell will contain all possible combinations If the two genomes are X and Y then (taking all virus particles as having 2 genomes as the simplest case) the progeny will include XX XY and YY genomes and the character of the fluid eventually obtained by inoculating a single XY particle will depend on a competition for selective survival amongst the various combinations We have evidence of such a process in the results of the cross M+/WS— in de embryonated eggs The ratio of MEL to WSE recombinants obtained is always strongly in favor of MEL the values ranging from 3 1 to 10 1 Since we can see no reason why these genomes should not be produced in equal numbers the discrepancy is best ascribed to the liberation of heterozygotes from the doubly infected cells and the subsequent major advantage of homozygous MEL over any other combination If all combinations are at random and all virus diploid the ratio of MEL WSE in harvested fluids from single particle infections would theoretically be 9 1

DR SABIN I would like to go back to the suggestion that Dr von Magnus made as an alternative explanation as to what happens after the use of large inocula that is that you get another type of variant instead of an incomplete virus The difference would be that the variant would still be able to multiply like the complete virus but it would not possess some of the other properties What I would like to ask is whether Dr von Magnus has used only one method of testing infectivity If we limit our host range to the chick embryo and if we limit the effect of infection to production of hemagglutinin no matter what method we use it is after all possible that if we had another mode of testing for the capacity of multiplication some of these virus particles might still be found to have the property of multiplication I say that because in our own work we have found that a poliomyelitis virus can lose its capacity to multiply in the mouse but still multiply in the monkey and do many other things Another thing that I want to bring up here is this that in the work with neurotropic virus hemagglutinins we found the very opposite phenomenon Thus Western equine encephalitis virus produces a good deal of hemagglutinin and has a high level of multiplication After several passages in mice less than six with large inocula we lose the hemagglutinin altogether—just the reverse On the other hand with small inocula hemagglutinin production will go on

longer In this case then we have lost the hemagglutinin property but have a variant which multiplies at a higher level than before the hemagglutinin was lost Now Dr von Magnus countered this suggestion by saying that if that were true why shouldn t such an influenza variant overgrow in five or six passages Well it may take longer I did ask some questions I hope Dr von Magnus

DR PREBEN VON MAGNUS Well concerning the first question testing other animals—we have of course titrated suspensions of incomplete and complete virus in mice too Actually it is much more fun to do it that way because you get a noted interference phenomenon in mice so you can actually detect your incomplete virus in suspension just from this autointerference We have not tried any other methods and I must confess that I do not believe that it will be possible to obtain a pure culture of incomplete virus even with continued passage We have tried to concentrate inocula from the fifth and sixth passage by high speed centrifugation hoping in that way to produce pure cultures of incomplete virus variants but we have never succeeded in it

MR BAWDEN With every plant virus that has been studied intensively during the last 10 years analogies have been found for the things Dr Magnus is calling incomplete viruses, We have however been careful not to use such a term for incomplete immature and such other words all carry implications that present knowledge cannot justify We have identified specific products in infected plants that resemble one another in various respects but differ in their ability to infect healthy plants and we have simply called our products infective or non infective The non infective particles of turnip yellow mosaic virus which lack nucleic acid might perhaps be aptly called incomplete but with most viruses we do not know why some particles lack infectivity A car with its brakes on will not move but that does not justify calling it incomplete Also with no plant virus is there any evidence to show whether the non infective particles are produced as stages in the synthesis of infective particles by the breakdown of infective particles or whether they are produced as such and never would have become or never have been infective particles Also and here perhaps I am making the same point as Dr Sabin because they do not infect healthy plants it cannot therefore be assumed that they had no biological activities in the cells where they were synthesized

Dr Luria has playfully likened infection by a phage to a woman going into a shop to buy a new coat I welcome similes when they are apt but this one seems to be simply misleading It is possible just to imagine a woman taking off her coat and throwing it away outside the shop but the next steps no What woman would enter a small shop to get a coat exactly like the one she has been wearing? And a shop stocking only one coat which

several hundred other women were also going to get? The infecting phage is not playing the role of a customer in a shop It is more in the position of a new owner who decides what the shop shall make and sell who designs the coats and has sufficient influence to ensure that they fit and are sold By infecting a bacterium the whole organisation of the cell is altered it comes under new management and produces new goods

7

Cellular Metabolism and Virus Growth

F C Bowden
Rothamsted Experimental Station Harpenden Herts England

The ability of plants to survive and grow in widely differing environments combined with their rapid responses to changes in illumination temperature and nutrition makes them excellent subjects for demonstrating how intimately all phases of virus diseases depend on the metabolism of the host cells Further as infections in many hosts are not lethal and they proceed uncomplicated by the concurrent formation of antibodies virus multiplication can be studied over unusually long periods

Against these favourable features must be set some disadvantages that complicate the interpretations of experimental results Prime among these is the need to wound leaves as a preliminary to infecting them there is no way of knowing particularly in the early stages of infection how many cells are infected and it is rarely possible to decide if an increase in the amount of extractable virus occurs because more has been produced in cells already infected or because more cells have become infected Another complication is that virus can be assayed only in extracts made by macerating infected tissues Errors can arise because a constant proportion of the virus may not always be extracted from the leaves because the method of assay may not detect all the specific particles present or because the process of extraction introduces artefacts Work with several different viruses has shown that extracts from infected leaves contain a range of serologically related particles not all of which are infective [8] The origin of the non infective particles is still in doubt With some viruses the ratio of non infective to infective material seems reasonably constant but with the Rothamsted tobacco necrosis virus it can vary widely with the treatments accorded to the extracts and with the condition of the leaves from which the extracts were made Infectivity tests also sometimes give misleading information about the extent of virus multiplication because extracts of many plants contain substances that inhibit infection

The multiplication of only a few of the known plant viruses has been *studied in any detail* but these suffice to show the dangers of generalisations and that there are great individual differences Almost the only common feature seems to be a period after infection before any new virus becomes detectable The length of this period, during which either no virus is detectable in the leaf extracts or the amount decreases with increasing time depends on the identity of the virus and host at about 20 it is less than 12 hours for Rothamsted tobacco necrosis virus in French bean and about 24 hours for tobacco mosaic virus in tobacco Once new virus becomes detectable it increases rapidly at first and then more slowly to reach a maximum concentration that varies enormously with different viruses and hosts It is perhaps worth noting here that virus host combinations which produce the largest yields of virus do not necessarily also display the most severe diseases With any one virus in the same host however differences in disease intensity more often than not are correlated with the amount of virus produced Many viruses reach higher concentrations in leaves that were well developed when they became infected than they do in those infected at the stage of buds

Present evidence suggests that the extent to which viruses accumulate *in vivo* is related to their stability *in vitro* Only viruses that are stable *in vitro* seem to reach high maxima which are then maintained over long periods Viruses that denature rapidly in leaf extracts usually reach much smaller maxima potato virus Y in tobacco for example only a thousandth or so that of tobacco mosaic virus Some unstable viruses occur in systemically infected leaves at about the maximum concentration over long periods but others decrease steadily in amount as the infected plants age Like the normal leaf proteins they are probably in a continuous state of flux so that their concentration reflects the balance between synthesis and degradation with the balance at any given time being set by the metabolism of the host cell Stable viruses such as tobacco mosaic seem less dynamic [0] However even when present in large quantities and not increasing in total amounts they seem not to be entirely static for their constitution can be affected by changing conditions in the host cells [14]

Most of the references in the literature to effects of changing host plant metabolism on virus diseases describe effects on symptoms I shall not be concerned with these except to say that the type and severity of symptoms can be altered out of all recognition by changing the conditions particularly of temperature and illumination under which infected plants are kept To take one example Around 20 tobacco mosaic virus causes in *Nicotiana glutinosa* only the necrotic local lesions that are commonly used in assays Above 30° infection is no longer lethal the plants become systemically infected and develop a yellow mottle When mottled plants that have been at 30 are placed at 20 they collapse and die within a day [18, 1]

Experiments to find how susceptibility to infection and how the extent of

virus multiplication are affected by changes in the state of host plants have been restricted to mechanically transmissible viruses that can be assayed quantitatively Most have been made with tobacco mosaic virus and how far the results apply at all generally is unknown Evidence is accumulating that different viruses multiply preferentially in different tissues Some leaf hopper transmitted viruses sugar beet curly top for example seem to occur predominantly in the phloem [10] by contrast some aphid transmitted viruses seem to occur predominantly in the leaf epidermis and tobacco mosaic virus more or less uniformly through the leaf [2] Such different distributions make it unreasonable to expect a uniform response to changes in the host. There is however abundant evidence with mechanically transmitted viruses that both the ease with which infection occurs and the extent to which the viruses multiply are closely related to the physiological state of the host There is much evidence too that the condition of cells that favours one process does not necessarily also favour the other Indeed the two can be affected so differently by changes in the host that it is difficult to avoid the assumption that some happenings essential for initiating infection differ fundamentally from those responsible for the synthesis of new virus Good examples of these differences come from experiments in which plants were kept under different conditions of illumination and temperature

Effects of Varying Illumination

If healthy plants are kept in darkness for a day or more before their leaves are inoculated with viruses their susceptibility as measured by the numbers of local lesions produced by a given inoculum may increase ten times Putting plants in the dark immediately they have been inoculated does not increase the lesion numbers but usually decreases them slightly [9] If the inoculum has been partially inactivated by exposure to ultraviolet radiation then the decrease may be considerable depending on the virus used With tomato bushy stunt or tobacco necrosis viruses but not with tobacco mosaic irradiated inocula may produce ten or more times as many lesions if the inoculated plants are exposed to daylight as if they are kept in the dark (Table 1) [7] As with bacteriophages [11] the infectivity of the irradiated plant viruses is not increased by exposing the inoculum to visible light Thus the increased number of infections occurs because of some light sensitive mechanism in the host cells whose operation decides whether or not particles already in the cell can act in a manner that allows them to be recognised as viruses There is no conclusive evidence to indicate whether it does so by reversing some change in the particles that was caused by the ultraviolet or whether it removes some barrier to infection so that damaged particles can become established

Visible light also favours virus multiplication and leaves inoculated with tobacco mosaic or tobacco necrosis viruses produce more virus per lesion if they are in daylight than if they are in the dark Many diverse

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Present evidence suggests that the extent to which viruses accumulate *in vivo* is related to their stability *in vitro*. Only viruses that are stable *in vitro* seem to reach high maxima which are then maintained over long periods. Viruses that denature rapidly in leaf extracts usually reach much smaller maxima, potato virus Y in tobacco, for example, only a thousandth or so that of tobacco mosaic virus. Some unstable viruses occur in systemically infected leaves at about the maximum concentration over long periods, but others decrease steadily in amount as the infected plants age. Like the normal leaf proteins, they are probably in a continuous state of flux, so that their concentration reflects the balance between synthesis and degradation, with the balance at any given time being set by the metabolism of the host cell. Stable viruses, such as tobacco mosaic, seem less dynamic. However, even when present in large quantities and not increasing in total amounts, they seem not to be entirely static, for their constitution can be affected by changing conditions in the host cells [11].

Most of the references in the literature to effects of changing host plant metabolism on virus diseases describe effects on symptoms. I shall not be concerned with these, except to say that the type and severity of symptoms can be altered out of all recognition by changing the conditions, particularly of temperature and illumination, under which infected plants are kept. To take one example: Around 20°, tobacco mosaic virus causes in *Nicotiana glutinosa* only the necrotic local lesions that are commonly used in assays. Above 30°, infection is no longer lethal, the plants become systemically infected and develop a yellow mottle. When mottled plants that have been at 30° are placed at 20°, they collapse and die within a day [16, 1].

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with viruses After some hours exposure to visible light the effect dis appears the epidermal cells regain their original susceptibility and pro duce lesions if again inoculated with virus[8] It is significant that virus in troduced during the refractory period when the normal nucleoprotein metabolism is presumably disturbed by the effects of exposure to ultra violet does not simply remain dormant and then proceed to multiply when conditions in the host again become favourable Apparently some step essential to infection cannot be postponed if it is not taken soon after virus enters a cell the particles seem to be destroyed or in some way rendered ineffective Some physiological states of the host encourage this first step which may be combination or fusion with some specific cell component others such as the changes caused by ultraviolet discourage it The varied substances that inhibit infection when present in inocula also may operate by discouraging this first step Their ability to prevent infection from occurring depends on the identity of the host plant inocu lated rather than on the identity of the virus and as some inhibitors seem not to affect the virus particles directly it seems they act by altering cell metabolism so that introduced virus is precluded from becoming estab lished[1]

Effects of Varying Temperature

Exposing plants to high temperatures has effects that somewhat re semble those produced by keeping plants in darkness When healthy plants are kept at 36 before they are inoculated their susceptibility to infection by all the viruses yet studied is much increased Also when plants are placed at 36 after inoculation fewer lesions occur than if they are placed at 20° but the effect is usually larger than by excluding light and it varies much more with individual viruses some of which regularly fail to cause any lesions at 36 (Table 2)[16] The ability of viruses to infect plants at 36 is not correlated with their resistance to heat *in vitro* as measured by the customary method of finding the temperature at which infectivity is lost after 10 minutes exposure Tobacco necrosis and tomato bushy stunt viruses which do not form lesions when inoculated plants are kept at 36 have thermal inactivation points of more than 80 as high or almost so as tobacco mosaic virus whereas tomato spotted wilt virus which forms lesions in plants at 36 has a thermal inactivation point of 45 It may be correlated with a low temperature coefficient of thermal inactivation for tobacco necrosis bushy stunt and cucumber mosaic viruses all share this property whereas tobacco mosaic virus does not However as neither tobacco necrosis nor bushy stunt viruses are inactivated in sap by 1 day at 36° it seems that the cell metabolism at 36 is such that some mechanism essential for the multiplication of these two viruses ceases to act or acts so poorly that the usual balance between breakdown and synthesis is drasti cally changed

Table 1

EFFECTS OF EXPOSING HOST PLANTS TO DAYLIGHT BEFORE AND/OR AFTER INOCULATION WITH UNTREATED AND WITH ULTRAVIOLET IRRADIATED VIRUS PREPARATIONS

		RTNV in French bean Before inoculation		*TBSV in N glutinosa Before inoculation*		*TMV in N glutinosa Before inoculation*	
		Dark	*Light*	*Dark*	*Light*	*Dark*	*Light*
Dark after	A	24	6 5	19 5	3	23	13
inoculation	B	14 5	2 5	[illegible]	0 7	10	8
Light after	A	24	11 5	84	7 5	50	12 5
inoculation	B	41	26	85	9	34	7

The numbers are mean numbers of lesions per leaf obtained on twelve to fourteen half leaves

A untreated virus at 1 mg /l RTNV (Rothamsted tobacco necrosis virus)
5 mg 1 TBSV (Tomato bushy stunt virus)
1 mg /l TMV (Tobacco mosaic virus)

B irradiated virus at 100 mg /l RTNV
500 mg /l TBSV
100 mg /l TMV

Light the plants were exposed to uncontrolled daylight

Dark the plants were kept in darkness for 24 hrs

mechanisms remain to be unravelled and we can do no more than guess at the reasons for light having such diverse effects depending on whether or not leaves are exposed to it before or after they are inoculated While in the dark products of photosynthesis and ascorbic acid will be decreasing and proteolysis will be encouraged the susceptibility of leaves to wounding when they are inoculated may also be increased Any or all of these may make it either easier for virus particles to enter epidermal cells when a leaf is rubbed or to become established as soon as they have entered Virus synthesis presumably calls for a source of energy and the increased production of virus by illuminating infected leaves may simply reflect the use of photosynthetic products as sources of energy

The light sensitive mechanism that allows ultraviolet damaged particles to infect is presumably the same one by which irradiated leaf cells counteract the damage ultraviolet causes to them It is of some interest that while repairing their own damage irradiated leaf cells resist infection by viruses to which they are normally susceptible Here again the metabolic state of the host cells determines whether or not introduced particles can multiply Leaves exposed to ultraviolet for short periods show no external signs of injury unless they are placed in darkness when their epidermal cells die If kept in daylight after irradiation an effect becomes evident only by the failure to obtain infection when the leaves are inoculated

its concentration may normally be maintained only by continued synthesis. Whether multiplication fails at 36° because the virus is directly inactivated by the high temperature, or because of a more indirect effect operating through some enhanced activity of the host-cells, is unknown. Whatever the cause, the practical consequence is that virus-free plants can be regenerated from parents systemically infected with some of the most heat-resistant viruses. It is an odd paradox that the only practical therapeutic treatment yet found for any virus diseases of plants should be one that is also most effective in increasing the susceptibility of healthy plants to infection.

Effects of Nutrition and Thiouracil

The nutrition of plants affects both their susceptibility to infection and the extent to which tobacco mosaic virus multiplies.[3,4] In general, both are increased by increased mineral nutrients if these also stimulate the growth of the plants. The effects of susceptibility to infection are small compared with those occasioned by changes in temperature and illumination, but effects on virus multiplication are considerable. In nitrogen-deficient plants, tobacco mosaic virus largely replaces the normal leaf proteins and in systemically infected leaves, particularly if they have abundant phosphorus, it may amount to more than two-thirds of the total protein. When extra nitrogen is provided, the virus-content of the leaves increases, but the normal proteins increase proportionally more, so that, although the virus concentration can be doubled, the ratio of virus to total protein may fall to less than 1 to 4. Many of the normal leaf proteins seem to be dispensable, in so far as the cell can still function when they are not produced or are produced in much smaller quantities than usual, and the synthesis of the virus takes precedence over these. Some workers have regarded the proteins that are replaced by the virus as virus precursors, but there is no evidence to support the view that any proteins of the healthy cells are transformed directly into virus. The only relationship between the two is that they are built from similar substances and that infection diverts some that would otherwise have gone into one kind of protein into another. Whether viruses that occur in much smaller amounts than tobacco mosaic virus produce comparable changes in the pattern of the normal proteins has not been studied. Nor is it known whether their multiplication would be similarly affected by changes in host nutrition, but the concentration of potato virus X in systemically infected plants was not consistently affected by fertilisers.[4]

Recent work on the multiplication of plant viruses has been done increasingly with detached inoculated leaves, or pieces of leaf, kept in water or solutions of various substances. This allows conditions to be more accurately controlled than when using whole plants and facilitates testing the effects of diffusible substances on virus multiplication. How far the

Table 2

EFFECT ON MEAN NUMBER OF LOCAL LESIONS OF KEEPING PLANTS AT 36° BEFORE OR AFTER INOCULATION WITH VARIOUS VIRUSES

Virus & host	*Plants at 36° Before inoculation* Control	1 day	2 days	*Plants at 36° After inoculation* Control	1 day	2 days
Rothamsted tobacco necrosis virus in bean	2	29	46	69	0	0
Tomato bushy stunt virus in *N. glutinosa*	18	83	97	65	9	2
Cucumber mosaic virus in tobacco	15	45	78	144	0	0
Tobacco mosaic virus in *N. glutinosa*	32	98	111	25	18	19
Tomato spotted wilt virus in tobacco	86	197	165	173	121	96

Prolonged exposure to high temperatures has long been used therapeutically to free some plants from systemic virus diseases[17] but nothing was known of the stability of the causative viruses and it was generally assumed they had low thermal inactivation points. It is now clear that the ability of viruses to survive in plants at high temperatures is no sure guide to their heat resistance *in vitro*. When plants systemically infected with tomato bushy stunt virus are kept at 36° the infectivity of leaf extracts falls steadily; the amount of material serologically related to the virus falls too, though more slowly than infectivity (Table 3).[18] This

Table 3

THE DECREASE IN VIRUS CONTENT OF SAP BY EXPOSING PLANTS SYSTEMICALLY INFECTED WITH TOMATO BUSHY STUNT VIRUS TO 36°

Time at 36°	*Precipitin titre of sap**	*Mean number of lesions per leaf with sap diluted* ½	1/10	1/100	1/1000
0 days	64	—	—	101	24
4 "	32	—	69	22	5
8	2	23	3	4	—
16 "	2	[illegible]	1	0	—

* Numbers are the reciprocals of the highest dilution at which the sap precipitated specifically with the virus antiserum

virus also loses infectivity *in vitro* at temperatures well below its thermal inactivation point but in these conditions the precipitin titre is unaffected. It seems then that host cells contain mechanisms that can break down even such a stable virus as tomato bushy stunt and at lower temperatures

Table 4

DIFFERENCES IN THE EFFECTS OF THIOURACIL ON THE MULTIPLICATION OF TOBACCO MOSAIC VIRUS IN LEAVES TREATED DIFFERENTLY

	Leaves kept in			
	Water		*Nutrient solution*	
Daylight	Water only	64	Nutrient only	197
	Thiouracil (100 mg 1)	8	Thiouracil	1
Darkness	Water only	48	Nutrient	1 8
	Thiouracil	32	Thiouracil	

Treatments were started 1 day after the leaves were inoculated and the leaves were macerated 6 days later Numbers are the reciprocals of highest dilutions at which clarified sap precipitated specifically with virus antiserum

No critical tests have been made but *virus already formed seems to be* unaffected and when the thiouracil is removed the virus again starts to increase

When mature healthy leaves are sprayed with or placed in solutions of thiouracil they show few or no signs that their metabolism has been much affected but the young leaves of growing plants become bleached and apical growth is stopped These effects appear not to be correlated with the failure of virus multiplication for they are not counteracted by applying uracil simultaneously with thiouracil That thiouracil can affect mature leaves is shown by applying it to inoculated leaves in which tobacco mosaic virus has already begun to multiply Normally the virus produces no lesions in inoculated tobacco leaves but when these are sprayed with thiouracil at different times after inoculation they develop necrotic spots and rings the size of which increases with increasing time between the time of inoculation and applying thiouracil Again there may be no relationship between this effect and failure of virus synthesis for the simultaneous application of uracil which allows virus formation to proceed does not prevent the formation of the necrotic lesions

Both thiouracil[1] and guanozolo[19] have been found to be incorporated in the nucleic acid of tobacco mosaic virus produced in leaves treated with them This discovery has naturally led to the suggestion that virus multiplication is inhibited because particles containing these foreign components are sterile There is however little evidence as yet that virus particles containing these substances do differ biologically from normal ones and the small amount of tobacco mosaic virus that is produced in leaves treated with thiouracil a day after inoculation is certainly not all noninfective Indeed weight for weight it seems not appreciably less

results from such experiments can be directly transferred to intact plants however is uncertain for placing leaves in water itself alters their responses to infection If leaves are immersed soon after they are inoculated infection occurs at fewer sites than if the leaves are left in air The proportional decrease depends on the state of the leaves for example young leaves of *Nicotiana glutinosa* are much less affected than old ones Infection seems not to fail in immersed leaves simply because virus particles diffuse from the inoculated cells into the ambient fluid but rather because the metabolism of the cells is changed for immersed parts of leaves produce more lesions if other parts are exposed to the air than if the whole leaf is immersed The number of infections is not affected if floating is delayed for a day or so after the leaves were inoculated so here again there is evidence of conditions that interfere only with some early stage in infection Unfortunately the interpretation of all such effects with inoculated leaves is uncertain The problem is to decide whether some entirely different kind of process is involved early in infection from those later involved in virus synthesis or whether the effects occur simply because early in infection the viruses are present only in the epidermal cells where their activities may be more readily interfered with than when they have reached less superficial cells So far from having any deleterious effect on the course of infection floating leaves a day after they are inoculated with tobacco mosaic virus increases the rate at which the virus multiplies Again the total amount of virus produced depends on the physiological condition of the infected leaves Leaves placed in solutions of sugar and phosphate may contain six days after infection four times as much virus as leaves placed in water and kept dark

Although many substances are known that inhibit infection when inoculated simultaneously with the viruses very few have been found to influence virus multiplication when applied to leaves after they have been inoculated Of these the most interesting are the pyrimidine and purine analogues of which thiouracil[11, 1] and guanozolo[18] have been the most studied We have used only thiouracil and have fully confirmed the fact that it can inhibit the multiplication of tobacco mosaic virus in tobacco leaves and that this inhibition can be counteracted by simultaneously applying uracil We have found however that the extent to which thiouracil inhibits virus formation depends a great deal on the physiological state of the infected leaves If the leaves are in conditions that encourage virus multiplication for example floated in nutrient solutions and exposed to daylight then thiouracil is very effective and almost stops virus formation but if the leaves are in conditions less favourable for virus multiplication for example if they are floated in water and kept dark then thiouracil only slightly reduces the rate of virus formation (Table 4) [6] Thiouracil can check the increase of tobacco mosaic virus at any time up to the peak of virus production but it has most effect when applied early in infection

need to do is become a part of the system that controls what is produced by the protein synthesising mechanisms of the cell On this view it is easy to account for the fact that so many treatments that facilitate the occurrence of infection do not also encourage virus multiplication Two quite distinct processes may be involved Establishing infection may entail linking or fusing the introduced virus or specific parts of it with the units that previously controlled the cell s metabolism producing a qualitative change comparable to acquiring some new genes Although virus production would be a consequence of this change it would be a very different process It would be one end product of protein synthesis by the cell the amount of virus produced would reflect the activity of this synthesis and the extent to which the new determinant influenced the character of the end products The view that viruses do not depend on the host simply for a supply of metabolites but also for the mechanisms by which they are synthesised fits present knowledge but I hope it will prove unfounded This is not because I have any philosophical prejudices in favour of viruses being organisms with independent activities but because I think the prospects of successful chemotherapy would be so much better if they were

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infective than virus formed in untreated leaves Virus particles are not the only components of infected cells in which these substances are likely to be incorporated and as thiouracil does disturb the host metabolism it is still reasonable to look for its action in this disturbance Particularly is this so as the action of thiouracil seems to depend on the physiological state of the host and any specificity in its inhibiting power seems to be determined by the host rather than by the virus It inhibits the multiplication of all the viruses we have tested in tobacco but of none in beans although one tested in beans was a tobacco necrosis virus which is inhibited in tobacco[5]

Such facts emphasize the importance of host cell metabolism in determining virus multiplication and they suggest that thiouracil interferes with this multiplication by influencing the synthetic mechanisms of the host but they do not prove it Other interpretations are equally plausible Virus particles that contain thiouracil may be able to multiply in one host but not in another Beans may contain more uracil than tobacco and tobacco leaves floated in water and kept in the dark may contain more than leaves floated in nutrient solution in the light so that they are better able to counteract the inhibiting action of thiouracil These things can be tested and no doubt answers will soon be obtained Our preliminary tests show that free uracil and other pyrimidines and purines disappear from leaf cells in which tobacco mosaic virus is being produced in quantity However there is no suggestion that virus multiplication either in normal leaves or those treated with thiouracil ceases because uracil is lacking Free uracil can be detected in extracts from uninfected leaves and from inoculated leaves in which thiouracil has prevented virus multiplication It is not detectable in extracts from leaves in which much virus has been formed but supplying extra uracil does not increase the amount of virus produced

Further studies on the action of such substances as thiouracil clearly promise to give much information on the mechanisms involved in virus multiplication and on its relationships with host-cell metabolism They may too disclose possibilities of chemotherapy for virus diseases I am not too optimistic about this however because I fear the viruses may have too few independent activities for them to be stopped by chemicals that do not also seriously disturb the host at least temporarily It is true that many diseases caused by bacteria and fungi are also greatly influenced by the physiological state of the host plants and nothing I have described necessitates the view that virus multiplication must differ fundamentally from the growth of other kinds of pathogens Nevertheless taken with the knowledge that all the plant viruses whose constitution has yet been determined are nucleoproteins it seems more reasonable to regard virus diseases as aberrations in the protein metabolism of the host than as the growth of independent parasites on a host[8] All that viruses may do or

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Plant Viruses and Proteins*

Barry Commoner
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Introduction

This paper describes investigations of the biochemistry of virus reduplication which are based on the special experimental advantages afforded by tobacco mosaic virus

The plant viruses of which tobacco mosaic is the best known are distinguished from other viruses by their relative simplicity Tobacco mosaic virus (TMV) is composed wholly of a specific protein and a pentose nucleic acid The biological properties of the virus (reduplication mutability action on the host) are necessarily derived from the whole or some part of this nucleoprotein For this reason the replication of a virus such as TMV can be dealt with as a process of nucleoprotein synthesis the initiation and specificity of which is determined by the entry of a virus particle into a suitable host cell In this sense TMV reduplication represented a virus induced change in the host s pattern of protein metabolism

The relatively simple composition of TMV and the ease with which the virus is isolated from infected tissue has led to quantitative description of the amino acid composition of the virus protein and the nucleotide composition of its nucleic acid It is possible therefore to specify the chemical requirements of any source material from which TMV is made

Given these and related advantages the following experimental strategy can be used as a means of describing the biochemical events which are involved in TMV reduplication The protein metabolism of otherwise identical uninfected and TMV infected tobacco leaf tissue is described quantitatively The processes specifically associated with TMV reduplication can be sorted out from the complex array of normal processes by comparison of the two sets of data From these metabolic differences and the con

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current determination of the amount of TMV synthesized inferences can be drawn concerning (a) the protein products of the biosynthetic processes set off by inoculation (b) the sources of nitrogen employed for virus synthesis and (c) the metabolic pathways which lead from initial source to final products The application of isotope tracer techniques to such an experimental system provides a ready means of confirming the conclusions derived from metabolic comparisons

Reported below are a series of investigations which are based on this approach From the results obtained it has been possible to describe in broad outline at least some of the biochemical events which occur when TMV is reduplicated in the cells of an infected leaf

Experimental

The source of nitrogen for synthesis of TMV proteins It is of obvious importance to know where the nitrogen used in the synthesis of virus protein comes from One approach to this question is afforded by nitrogen balance experiments in which the overall nitrogen economy of otherwise identical infected and uninfected tissue is compared

Such experiments can be carried out by preparing uninfected tissue from one half of a large normal tobacco leaf and infected tissue from the opposite leaf half Discs of leaf blade are then cut from the two leaf halves and are cultured in an inorganic medium under constant conditions Comparable infected and uninfected samples are removed from culture at daily intervals after the time of inoculation fractionated to yield various nitrogenous components (including TMV itself) which are then determined quantitatively by suitable means The difference in the levels of the various nitrogen fractions in infected and uninfected tissue when compared with the time course of virus synthesis leads to conclusions concerning the relationships between TMV synthesis and various aspects of nitrogen metabolism

Detailed experiments of this type have been previously reported [1-8] and only the major conclusions will be described here It was first observed[1] that during the time of TMV synthesis there is a reduction in the relative amount (i e as compared with uninfected controls) of non protein nitrogen present in infected tissue Since the major component withdrawn during TMV synthesis was free ammonia and since the changes in other non protein nitrogen components (i e free amino acids and amides) appeared to be consequences of the ammonia withdrawal it was suggested that ammonia is the ultimate source of TMV protein nitrogen This proposal was confirmed by isotope experiments[3] which showed unequivocally that the nitrogen of TMV protein must be derived from tissue ammonia These results also showed that the isotope content of the free amino acids and amides was too low to permit these components to act as intermediates between ammonia and TMV protein nitrogen

This evidence necessitated the somewhat unexpected conclusion that TMV protein nitrogen is not synthesized by means of peptide condensation of the requisite *free* amino acids It should be emphasized that the data do not exclude the possible formation of TMV protein peptide bonds in the commonly accepted manner from amino acid amino and carboxyl groups The data show only that if this does occur the amino acids involved are not in equilibrium with the *free* amino acids but are somehow sequestered from them perhaps by being bound to the surface of a protein or cell particulate The diversion of normal protein synthesis which in the long run leads to the formation of TMV protein begins at the very lowest bio

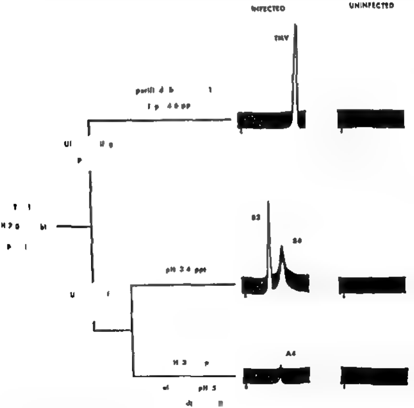

FIG 1 Ascending electrophoretic patterns obtained from comparable fractions of soluble protein extracts of uninfected (right column) and systemically infected (left column) leaves of *N tabacum* (variety White Burley) Movement is to the right All preparations were in 05 M phosphate buffer pH 7 0 Electrophoresis was carried out for 90 minutes at an average current of 8 5 ma Ultracentrifugation was carried out for one hour at 104 500 × g

chemical level, with the incorporation of ammonia into products which are specific precursors of the virus protein

The proteins synthesized in infected tissue TMV is itself the chief new product of the altered path of protein synthesis induced by infection It is of interest to know whether TMV is the exclusive new protein formed or whether other non normal proteins have even a temporary existence in infected tissue An understanding of the number and nature of the products is essential to any analysis of the new protein synthesizing system brought into action by the inoculation of a tobacco leaf with TMV Also important is the possibility that small amounts of non virus proteins not found in normal leaf may signify the occurrence of protein precursors of TMV

In order to investigate these questions we have compared the soluble proteins occurring in normal and TMV infected tobacco leaf The chief results are summarized in Figure 1 which shows electrophoretic patterns

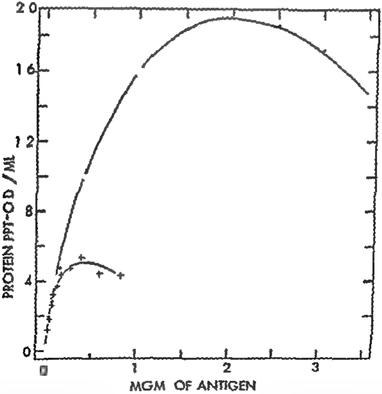

FIG 2 Precipitin reactions of TMV and protein B8 with rabbit sera prepared against electrophoretically homogeneous TMV Precipitate formed by reacting 05 ml of anti TMV serum with indicated amount of TMV (points upper curve) and B8 (plus signs lower curve) Optical densities are at 750 mμ and represent color due to Folin phenol reaction of precipitates in 1 ml of reagent

of comparable protein fractions isolated from infected and uninfected leaf In addition to TMV infected leaf contains three other proteins not found in normal tissue All of these are low molecular weight proteins ($S = 3$) All lack nucleic acid None of the three proteins is infectious Two of the nonvirus proteins (B3 and B6) precipitate at pH 3 4 the third (A4) is soluble at this pH The three proteins are readily distinguished by their electrophoretic mobilities

The three nonvirus proteins are close immunochemical relatives of TMV As shown in Figure 2 protein B8 (a polymerized form of B3) cross-reacts with rabbit serum prepared against highly purified TMV the reciprocal type of cross reactivity between TMV and anti B8 serum is shown in Figure 3 Similar tests show that all three nonvirus proteins will react with sera prepared against TMV and protein B8 These close immunochemical

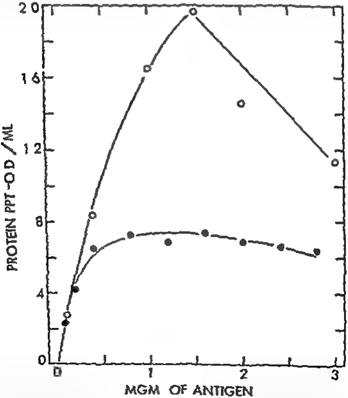

FIG 3 Precipitin reactions of TMV and B8 with anti B8 serum Precipitate formed by reacting 05 ml of anti B8 serum with indicated amounts of TMV (open circles upper curve) and B8 (closed circles lower curve) Ordinate as for Figure 2

chemical level with the incorporation of ammonia into products which are specific precursors of the virus protein

The proteins synthesized in infected tissue TMV is itself the chief new product of the altered path of protein synthesis induced by infection It is of interest to know whether TMV is the exclusive new protein formed or whether other non normal proteins have even a temporary existence in infected tissue An understanding of the number and nature of the products is essential to any analysis of the new protein synthesizing system brought into action by the inoculation of a tobacco leaf with TMV Also important is the possibility that small amounts of non virus proteins not found in normal leaf may signify the occurrence of protein precursors of TMV

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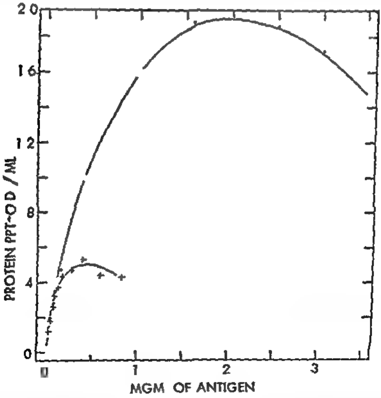

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It had been observed previously[3] that the fraction of soluble leaf protein which included proteins B3 and B6 becomes heavily labelled with N^{15} in isotope experiments thus indicating that these proteins like TMV proper are synthesized *de novo* in infected tissue To analyze these relationships further experiments have been carried out in which the time-course of formation of TMV and proteins A4 B3 and B6 was followed quantitatively For this purpose it was convenient to use the immunochemical reactions of the nonvirus proteins The results of one such experiment are shown in Figure 4A (upper curve) It is evident from these results that the nonvirus proteins are not synthesized in parallel with TMV but appear suddenly only after about one third of the final amount of virus has been attained Since the nonvirus proteins appear abruptly at about 200 hours after inoculation it seems likely that they occur as a result of some qualitative change in the character of the TMV-synthesizing apparatus at that time

The chief conclusions to be derived from our investigations of the nonvirus concomitants of TMV reduplication are that (a) The protein synthesizing apparatus newly activated by the inoculum produces a multiplicity of related proteins (b) The system responsible for TMV replication undergoes an apparently abrupt change in character partway through the infection process (c) One consequence of this change is the appearance in the tissue

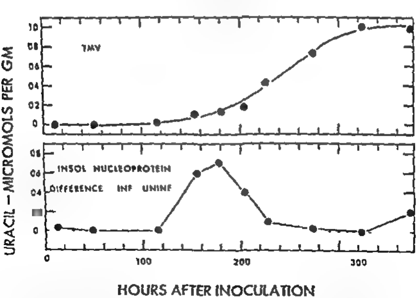


FIG 4B Experiment of Figure 4A Upper curve represents the uracil content of the TMV isolated at various times after inoculation Lower curve represents the difference between infected and uninfected leaf with respect to the uracil content of insoluble pentose nucleoprotein

inter relationships among TMV and the nonvirus proteins are evidence that the four proteins possess some common structural properties and perhaps a common origin

These results add significantly to our meager knowledge of the changes in host protein synthesis which are associated with virus reduplication The occurrence in infected leaf of three nonvirus proteins absent from uninfected tissue imposes new requirements on any proposal which purports to de scribe the mechanism of TMV reduplication Any explanation of TMV reduplication must also account for the occurrence properties and metabolic behavior of these non infectious concomitants of the reduplication process

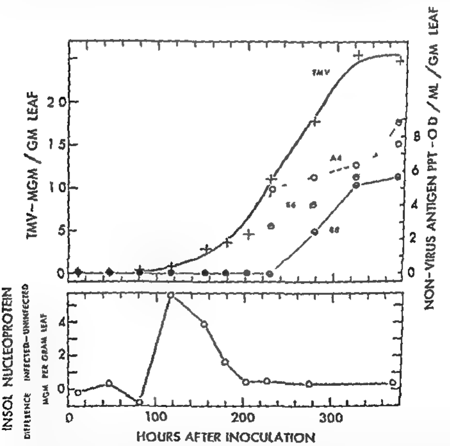


FIG 4A The amount of various proteins found in nutrient-cultured tobacco leaf tissue at various times after inoculation with TMV Virus determined chemically Proteins A4 B6 and B8 determined immunochemically as described in text The lower curve represents the differences between the nucleoprotein extractable with 10% NaCl solution from insoluble fraction of infected tissue and comparable uninfected samples Values given are based on microKjeldahl nitrogen determinations

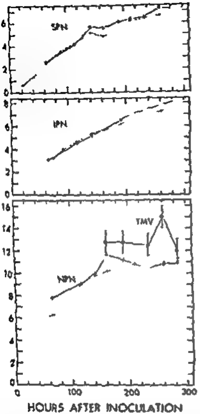


Fig 5

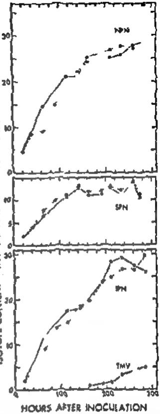


Fig 6

Fig 5 Changes in atom % N^{15} in TMV non protein nitrogen (NPN) insoluble protein (IPN) and soluble protein (SPN) of uninfected tobacco leaf discs (broken lines) and TMV infected discs (solid lines) during culture in N nutrient containing 30 atom % N^{15} Values for NPN IPN and SPN obtained without dilution with carrier nitrogen mean error less than 001 atom % N^{15} Values for TMV based on 1 10 dilution with carrier nitrogen mean error about ± 1 atom % N^{15} (represented by vertical lines through points)

Fig 6 N^{15} content of various fractions data derived from experiment of Figure 5 Open circles broken lines uninfected discs Closed circles solid lines infected discs

insoluble residue with 10% NaCl varies during the infection process When the differences in the amount extractable from comparable uninfected and infected tissue are determined at various times after inoculation the lower most curve of Figure 4A is obtained This shows that infected tissue devel ops an excess in this insoluble protein fraction which reaches a maximum at about 120 hours and declines to zero at about 220 hours

Fractionation experiments with the protein extractable in 10% NaCl

of nucleic acid free non infectious proteins which are close immunochemi cal relatives of TMV

The insoluble proteins involved in TMV reduplication In early studies of the overall nitrogen economy of infected tissue it was found that an excess (as compared with controls) in insoluble leaf protein appears soon after inoculation[8] The significance of this effect was revealed by subsequent isotope experiments If N^{15} enriched nutrient me dium is used in a standard nitrogen balance experiment data are obtained which permit comparison of the metabolic behavior of protein fractions in infected and uninfected tissue Figure 5 shows the time course of change in N^{15} enrichment of 4 fractions (TMV non protein nitrogen soluble protein nitrogen and insoluble protein nitrogen) from infected and uninfected leaf discs obtained from opposite halves of the same tobacco leaves The curves show that significant differences in N^{15} incorporation are associated with the infection process This is more apparent from Figure 6 in which the data have been recalculated to show the actual amount of N^{15} contained in each fraction at various times after inoculation These curves show that isotopic nitrogen is withdrawn from the non protein nitrogen pool when TMV is synthesized This confirms our previous observation that ammonia is withdrawn from the non protein nitrogen pool during TMV protein syn thesis Furthermore the data show that just *before* isotopic nitrogen is converted to TMV protein an extra amount of N^{15} which is about equal to that finally incorporated into TMV in this experiment (5 micrograms of N^{15} per 100 mg of tissue) is taken into the insoluble protein fraction of the infected leaf A little later when the isotopic nitrogen begins to appear in the virus this excess N^{15} is withdrawn from the insoluble protein frac tion These effects are shown graphically in Figure 7 which represents cal culations of the actual amounts of isotope which enter and leave the insol uble protein fraction It is evident from these results that nitrogen ultimately appearing in TMV protein is first withdrawn (as ammonia) from the non protein nitrogen pool then incorporated into the insoluble protein and finally transferred from this fraction to TMV itself It must be con cluded that some component included in the insoluble protein fraction represents the site at which TMV protein is synthesized

This conclusion has caused us to turn our attention toward an analysis of the events which take place in association with this insoluble protein As an initial step in this direction it could be shown that the isotope effects involve a relatively small part of the insoluble protein fraction which is characterized by a specific gravity[3] in the range 1 00–1 17 This rules out the chloroplast as the locus of the observed effects

The insoluble protein fraction is defined by the fact that it will not dis solve in phosphate buffer (pH 7 0) of low ionic strength However if this fraction is extracted with a 10% NaCl solution a considerable amount of protein is dissolved The amount of protein which is extractable from the

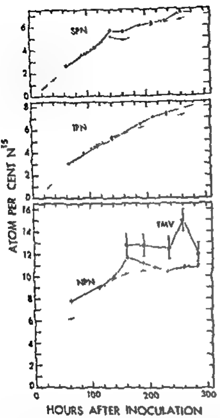
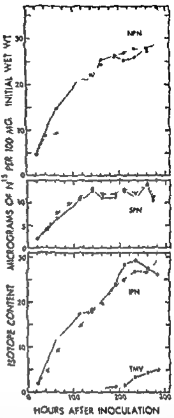


Fig 5



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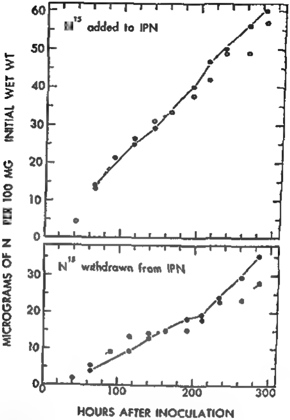

FIG 7 Amounts of N^{15} incorporated into and withdrawn from IPN fraction Open circles broken lines uninfected discs Closed circles solid lines infected discs

shows that the observed differences between infected and uninfected tissue are due in part to a pentose nucleoprotein Experiments on the nucleic acid economy of infected and uninfected tissue provide additional insight into this problem In the experiment described in Figure 4A the nucleic acid present in the insoluble protein fraction was extracted hydrolyzed to the N bases and analyzed by paper chromatography The results showed that an excess in insoluble PNA developed for a time early in the infection process The amount of this excess in insoluble PNA found at various times after inoculation parallels the excess in insoluble protein quite closely The best evidence for this effect is obtained from the uracil data since these are unequivocally due to PNA and are not affected by possible contamination from DNA These results are presented in Figure 4B This curve which shows the difference in insoluble PNA uracil found in infected and un infected tissue at various times after inoculation leads to the following conclusion

Like the insoluble protein excess insoluble PNA uracil appears in in fected tissue before TMV appears The excess reaches a maximum at about the time that TMV formation starts Then as TMV appears the uracil excess disappears reaching zero at about 220 hours Significantly the max imum uracil excess (07 μM/g of tissue) is of the order of magnitude of the amount of uracil which appears in the TMV finally synthesized (1 μM/g) this is shown in Figure 4A This result suggests that the excess insoluble uracil is incorporated as such into TMV nucleic acid

It now appears likely that the nucleic acid ultimately incorporated into TMV is synthesized previous to formation of the entire TMV particle as a buffer insoluble pentose nucleoprotein

Interpretations and Conclusions

The conclusions which are *required* by the above data may be sum marized as follows

1 When tobacco leaf is inoculated with TMV the course of protein synthesis is so altered as to result in the appearance of at least four buffer soluble proteins never found in normal leaf These are TMV nucleopro tein and the nucleic acid free non infectious low molecular weight pro teins A4 B3 and B6

2 The proteins newly synthesized in infected leaf appear to be formed *de novo* It can be shown that the bulk of the nitrogen found in the protein of TMV is derived from free ammonia of the host without passage through the pool of free amino acids

3 These observations require the conclusion that TMV protein is not formed by peptide condensation of the requisite *free* amino acids Virus protein *may* be formed by peptide condensation of amino acids in the order in which the residues occur in TMV but if this occurs the amino acids in volved must be sequestered from the free amino acid pool and must be synthesized at a particular site as a result of a process specifically induced by infection Hence the diversion of host protein metabolism induced by infection begins at the lowest biochemical possible level at which ammonia nitrogen becomes associated with the carbon residue required to form the necessary amino acid configurations

4 The initial phases of the protein synthesis due to TMV infection take place in association with a buffer insoluble protein which is possibly a cell particulate There is preliminary evidence that this initial phase involves the primary formation of TMV nucleic acid followed by formation of virus protein

5 The occurrence of three nonvirus proteins which are immunochemi cally related to TMV shows that TMV reduplication must involve processes considerably more complex than the direct replication of the virus particles which enter the cell as inoculum These proteins are not continuous prod ucts of TMV activated protein synthesis in the leaf but are found after an

abrupt qualitative change in the new path of protein synthesis takes place The fact that the time of this change coincides with the time at which the previously synthesized excess of PNA in the infected leaf runs out suggests but by no means proves a causal relationship between nucleic acid depletion and the appearance of the nonvirus proteins

So much for the conclusions which the data demand It is appropriate however to consider at this point another class of possible conclusions concerning the process of TMV reduplication These while not demanded by the data are consistent with them This type of conclusion originates not in the data but in the desire to affirm or deny a particular conception of virus reduplication

Take for example the question of possible protein precursors of TMV Our data as they stand at present do not *require* the conclusion that any protein which we have isolated is in fact a precursor of TMV Nor does our very meager knowledge of protein synthesis in general even require that such precursors exist There is no reason at present to deny the possibility that TMV protein is formed in one Jovian blow from ammonia and say the keto acids required to make the necessary amino acid residues In other words the idea that TMV does in fact have a detectable precursor is not demanded by our knowledge of the synthesis of TMV or of plant protein synthesis in general

Where then does such a concept originate and what bearing does it have on the problem of TMV synthesis? It is not difficult to see that the notion of a virus precursor originates in the generalized experience that the biosynthesis of many substances does indeed proceed by way of the initial formation and later conversion of a substance which represents some basic part of the final product

It is our preference to refrain from drawing conclusions of this type Nevertheless the temptations in this direction are severe To illustrate this point we may examine some further data dealing with protein B8 This protein is formed by irreversible polymerization of protein B3 Might it be a precursor of TMV i e TMV less its nucleic acid? If electron micrographs of B8 and TMV are compared (Figure 8) a close resemblance of the two proteins is apparent

A superficial interpretation of this result might lead to the notion that B8 represents TMV minus its nucleic acid This conclusion might lead to the further suggestion that the addition of nucleic acid derived from TMV to protein B8 might actually accomplish an *in vitro* synthesis of TMV from these component parts Like many proteins B8 will in fact combine with nucleic acid prepared from TMV yielding an artificial nucleoprotein with an ultraviolet absorption spectrum quite similar to that of the virus (Figure 9) The complex is not infectious and it is of course rather presumptuous to suppose that the mere mixing of two ingredients would so readily yield an infectious virus particle

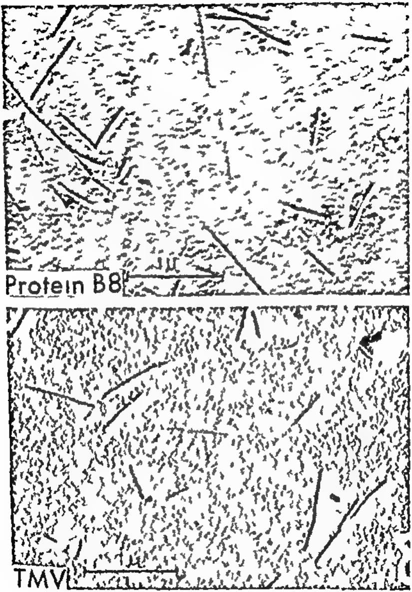

Fig 8 Chromium shadowed electron micrographs of TMV and protein B8 obtained from pH 7 0 solutions of these proteins

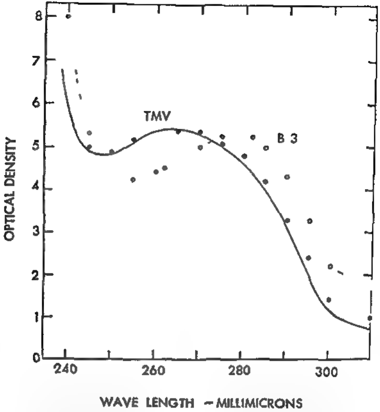

FIG ■ The ultraviolet absorption spectra of protein B8 (open circles dotted line) TMV (solid line) and a complex of protein B8 with nucleic acid prepared from TMV (closed circles)

Despite these superficial parallelisms between the properties of B8 and TMV there ■ as yet no real reason to conclude that B8 is in fact a pre cursor of TMV On the contrary from preliminary amino acid analysis which reveals the presence in B8 of two amino acids not found in TMV the simple conclusion that B8 ■ TMV less its nucleic acid appears ■ be un warranted

These points are made in order to delineate the extent to which extrapolations from our data are unwarranted Our experiments have told us a good deal about the biochemistry of TMV synthesis but by no means enough We do have ■ set of isolated pictures which describe several inter esting aspects of TMV reduplication It ■ tempting to spread our conclu sions from these few islands of fact to cover ■ least in imagination the still unknown areas which lie between But it ■ perhaps wiser to wait and to work for more information

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9

Abortive Infection with Viruses *

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When a virulent phage particle adsorbs to a susceptible host bacterium it forms a new entity the infected bacterium which possesses properties distinct from those of either of its two components The infected bacterium shares with the phage particle the property of producing a plaque when mixed with susceptible bacteria on an agar plate being called for this reason an infectious center The total number of infectious centers present in a phage host cell mixture includes the productive infected bacteria and any unadsorbed phage particles

The infected bacterium retains its plaque forming potential throughout the latent period of intracellular virus multiplication At the end of the latent period it lyses liberating a number of plaque forming phage particles This normal course of events can be interrupted by a number of environmental factors which cause ultimately a destruction of the plaque forming potential of the infected bacterium This inactivation of the infected bacterium can occur under conditions which *do not destroy* the unadsorbed phage particle or the uninfected bacterium The phenomenon has been called *abortive infection* because under these conditions infection does not result in the production of viable phage progeny

In spite of the obvious chemotherapeutic possibilities inherent in the increased sensitivity of infected host cells to destructive agents there has been no systematic study of this property The purpose of this paper is to assemble from the literature various examples of abortive infection to see if any generalizations may be drawn concerning this phenomenon For purposes of discussion abortive infection is defined as the loss of the plaque forming potential of infected bacteria under conditions which do not result in destruction of either free phage or uninfected bacteria It is realized that

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this definition may result in the grouping of many mechanisms of inactivation into one category

One of the first recognized examples of a chemical agent capable of causing abortive infection was 5-methyl tryptophane[7,8] The addition of this tryptophane antimetabolite at the time of infection resulted in a rapid destruction of infectious centers starting at the end of the normal latent period The antimetabolite had no lethal effect on either free phage or uninfected bacteria Similar results were obtained with methionine sulfoxide an antimetabolite for glutamic acid[15] These results indicate that the interruption of protein synthesis in phage infected bacteria causes an irreversible destruction of their plaque forming potential

Another group of compounds which cause abortive infection includes the inhibitors of energy metabolism such as cyanide and dinitrophenol The experiments which have been reported with cyanide have been rather contradictory Doermann[10,11] working with *E coli* infected with phage T4r found that the addition of cyanide during the first half of the latent period resulted in a gradual loss of infective centers without bacterial lysis The same result was reported for T3 infected bacteria by Anderson and Doermann[3] The medium used was chemically defined containing glycerine as energy source Cohen[6] found that the addition of cyanide or iodoacetate to bacteria infected with T2r+ or T4r+ in a lactate medium resulted in a prompt lysis of the infected bacteria without release of viable phage Lysis did not occur under these conditions with bacteria infected with phage T7

In contrast Benzer and Jacob[5] reported that the addition of cyanide to broth grown bacteria three minutes before infection with T2 or phage λ resulted in prevention of phage development but with no loss of infectious centers during one hour at 37 C From these various reports it would appear that the effect of cyanide on infected bacteria is a function of the genetic constitution of the phage and possibly of the composition of the growth medium

The effect of 2 4-dinitrophenol (DNP) on phage infected bacteria was studied by Heagy[17] The addition of DNP at the time of infection of *E coli* with phage T2r+ resulted in prompt lysis of the infected bacteria without liberation of viable phage The results were the same whether the bacteria were grown in nutrient broth or in synthetic medium with glucose In contrast the addition of DNP to bacteria infected with T2r or with T1 *inhibited* normal phage lysis With phage T2r there was a slow loss of infectious centers Free phage was not inactivated by DNP These results as far as they go are very similar to those reported by Cohen and by Doermann with cyanide

Under certain conditions anaerobiosis has the same effect as the addition of cyanide or DNP[6] Bacteria in a chemically defined lactate medium were made anaerobic by passage of a stream of nitrogen to displace the air On infection with T2r+ there was prompt lysis with loss of infectious centers

In contrast infected bacteria in the same medium under aerobic conditions did not lyse (Lysis inhibition with r+ phage) In this medium energy is supplied only by aerobic oxidation of lactate

Monod and Wollman[21] observed a prompt initiation of lysis of phage infected *E coli* in a lactose containing medium in which the lactose was not available because the cells were not lactose adapted This was confirmed by Heagy[17] using T2r+ infected *E coli* Heagy found also that prompt lysis of T2r+ infected cells occurred in a chemically defined medium lacking any energy source As pointed out by both Cohen and Heagy the common factor in all these cases of unproductive lysis of infected bacteria is the absence of an energy supply

A different type of destruction of infectious centers is brought about by the drug proflavine [14] The bacteria were grown in a chemically defined medium and proflavine was added two minutes before phage infection There was no loss of infectious centers during the latent period but at the end of the latent period there was a rapid lysis of the bacteria without liberation of viable phage The time relationships suggested that some terminal step in phage maturation was prevented This suggestion was confirmed by DeMars Luria Fisher and Levinthal[9] who demonstrated that lysis of proflavine treated cells resulted in release of incomplete phage particles These particles were shown to be the protein shells of phage heads lacking the nucleic acid core and the tail This destruction of infectious centers occurs under conditions in which free phage and uninfected bacteria are stable The chemical reaction which is inhibited by proflavine is not known

Aureomycin is another drug which brings about abortive infection The effect of aureomycin on *E coli* strain B infected with phage T3 was studied by Altenbern He found that infected bacteria were inactivated much more rapidly than uninfected bacteria while free phage was resistant to the drug Cationic detergents increased the activity of the aureomycin Certain aspects of the action of aureomycin on infected bacteria resembled effects shown by proflavine but not enough information is available for an adequate comparison of the two drugs

Another mechanism of destruction of infectious centers is found in the case of certain calcium requiring bacteriophages Phage T5 adsorbs to its host cell in the absence of calcium but phage is not produced unless calcium is added [1] Incubation of infected bacteria in a calcium free environment results in a progressive loss of infectious centers beginning soon after adsorption These experiments were confirmed with phage T5 and duplicated with a typhoid phage by Fildes Kay and Joklik [13] The time relationships had indicated that calcium was involved in an early stage in phage infection Lanni and Luria [0] later demonstrated that calcium was essential for the injection of phage T5 DNA into the host cell In the absence of calcium the phage adsorbs to kills and causes cytological changes in the host cell behaving somewhat like the ghosts of phage T2 [18] When damage

to the host cell becomes severe enough it is unable to support phage growth even though the addition of calcium would make injection possible

What may involve a similar mechanism is the inactivation of phage T1 on adsorption to its host cell in buffer Phage T1 when mixed with host bacteria in dilute phosphate buffer at 37 C appeared to be inactivated immediately on adsorption to the bacteria However if the phage was incubated with calcium ion or with broth for a short time before adsorption normal infected bacteria were produced with no loss of infectious centers In this case also it would appear that the divalent cation is essential for some stage in the infectious process subsequent to adsorption

This phenomenon seems to have been rediscovered by Joklik[19] who studied the adsorption of phage T1 in various media The proportion of active adsorptions was markedly a function of the ionic environment being increased by magnesium and calcium ions

Another variety of abortive infection was discovered by Benzer[4] Strain B of *E coli* was grown in broth washed with buffer and aerated in buffer for one hour at 37 C to exhaust intracellular nutrients The starved bacteria adsorbed phage well but did not lyse and did not liberate phage progeny If the infected starved bacteria were transferred to broth at 37 C phage development began immediately The latent period timed from the addition of broth was about 10% shorter than under normal circumstances Benzer noted that ⅓ to ½ of the viable phage particles which became adsorbed to starved bacteria failed to form infectious centers No study was made of the fate of these missing phage particles

A more extensive study of this kind of abortive infection was made by Gross[16] The host cells were strain K12 of *E coli* grown in a chemically defined glucose salts medium washed in buffer and starved by aeration at 37 C Coli phage T2 was adsorbed to the starved host cells at 37 C and the adsorption mixture was assayed at intervals for total infectious centers and for unadsorbed phage At each time interval only 60% of the infected bacteria were infectious centers the 40% deficit being termed abortive infection Abortive infection does not occur with unstarved cells and increases with the duration of starvation reaching 80% or more of the cells infected after three hours of starvation Starved K12 cells when incubated in broth or defined medium for ½ hour before infection recover completely and give no abortive infection The proportion of a starved bacterial population giving abortive infection is independent of the number of phage particles adsorbed per cell so that the probability of a given infected cell producing phage progeny is not increased by infecting it with more than one phage particle

In these experiments adsorption of phage was normal and every infected bacterium was killed by the adsorbed phage whether it resulted in abortive infection or not Turbidimetric studies indicated that the bacteria giving abortive infection were lysed before the middle of the normal latent period

while those infected bacteria yielding phage progeny lysed at the end of the latent period These results with starved bacteria are similar to the effects reported with cyanide and dinitrophenol and discussed above

Gross also studied the properties of the infected starved bacteria which did not undergo abortive infection Under appropriate conditions about ½ of the infected bacteria would be abortively infected and the remainder would form normal infectious centers which on transfer to broth would lyse at the end of the latent period with a normal yield of phage progeny These infectious centers were stable for several hours in iced buffer but were fairly rapidly inactivated in buffer at 37 C Incubation of infectious centers in the chemically defined glucose salts medium resulted in an accelerated inactivation as compared with buffer alone at 37 C Addition of yeast extract or a mixture of amino acids to the chemically defined medium resulted in normal phage development in the starved infected bacteria with no loss of infectious centers No single specific nutritional factor could be found which was essential for the stabilization of the infectious centers It seemed that a deficiency of amino acids in the starved bacteria resulted in this type of abortive infection so the mechanism may be similar to that of abortive infection produced by 5–methyl tryptophane or methionine sulfoxide

While studying the nutritional role of calcium in the multiplication of coliphage T5 I found yet another example of abortive infection which resembles in some respects the case studied by Gross Strain B of *E coli* was grown in broth washed in buffer and starved by aeration in buffer at 37° C for one to two hours The buffer contained 0 1M NaCl 0 001M Na_2HPO_4 0 001M $CaCl_2$ and 0 001M $MgSO_4$ and was adjusted to pH 7 2 Unadsorbed phage T5 and uninfected starved bacteria were stable in this buffer for hours at 37 C Phage T5 was permitted to adsorb to the starved bacteria in this buffer at 0 C There was no loss of infectious centers during adsorption and the infected bacteria were stable at 0 C for several hours at least

If the infected bacteria were diluted into broth at 37 C there was no loss of infectious centers and the bacteria lysed at the end of the normal latent period liberating a good yield of phage If the infected bacteria were diluted into buffer at 37 C there was an exponential destruction of infectious centers beginning at the time of temperature change the half life of the infectious centers being about 7 minutes The rate of inactivation varied with the temperature of incubation Over the range from 20 to 37 C the activation energy (Arrhenius Constant) for the inactivation process was about 13 000 calories corresponding to a Q_{10} of about 2 3 This temperature coefficient is typical of many enzyme catalyzed reactions and suggests that the inactivation may be the result of enzyme action The killing of uninfected bacteria or unadsorbed phage particles is characterized by an activation energy of the order of 100 000 calories which is typical of protein denaturation These facts suggest that the destruction of infected bacteria

must involve a different kind of reaction from that responsible for the kill ing of bacteria and viruses by heat

The marked difference in behavior of infected starved bacteria when diluted into buffer or into broth at 37 C suggested that some nutritional factors in broth might stabilize the infected bacteria The addition of ammonium chloride and glucose to the buffer makes it a complete medium as far as the multiplication of *E coli* is concerned However this glucose–ammonia–buffer medium resulted in a somewhat more rapid inactivation of infected starved bacteria than occurred in buffer alone In contrast the addition of an acid hydrolysate of casein resulted in normal phage reproduction This suggested that the addition of amino acids to the buffer might result in stabilization of the infected bacteria

The presence of single amino acids such as leucine isoleucine or valine at a concentration of 0 1 mg /ml of buffer resulted in a marked slowing of the rate of inactivation of infectious centers Various mixtures of amino acids were more effective than single amino acids in stabilizing the infected bacteria It was possible to find amino acid mixtures in which the infected bacteria were stable at 37 C for more than an hour without detectable phage reproduction This suggests that it is possible to inhibit the lytic process without having all nutrients essential for phage reproduction pres ent in the environment and hence to study these two processes separately

Summary

Under certain environmental conditions phage infected bacteria are far more vulnerable to destruction than are either uninfected bacteria or unad sorbed phage particles This inactivation of infectious centers has been termed abortive infection because phage progeny are not produced It oc curs at physiological temperatures but not at 0 C The temperature coefficient in the one case where this has been studied is typical of an enzyme catalyzed reaction not of protein denaturation In some cases at least the destruction of infectious centers is accompanied by lysis of the infected bacterium

Abortive infection occurs when normal phage development in infected bacteria at physiological temperatures is prevented by any of a number of different mechanisms The known causal factors include

1 interruption of energy supply by cyanide dinitrophenol anaerobiosis or starvation

2 interference with amino acid metabolism by use of antimetabolites such as 5–methyl tryptophane or methionine sulfoxide and by starvation

3 prevention of phage development by means of drugs of unknown mechanism such as proflavine and aureomycin

4 prevention of phage development by removal of calcium ion an essential growth factor for certain phages

The simplest explanation for the phenomenon of abortive infection is that infection of a host bacterium by a phage particle initiates two distinct processes which may proceed simultaneously but more or less independently One of these the reproductive process leads ultimately to the production of phage progeny The other the lytic process results in disorganization of the host cell and ultimately in cell lysis In the usual course of events the reproductive process is interrupted at the end of the latent period by bacteriolysis the burst size being the average number of phage particles which have been completed up to the time of lysis In abortive infection the reproductive process is inhibited but the lytic process occurs and may even be accelerated under certain circumstances Chilling to 0° C prevents both reproductive and lytic processes from occurring The phenomenon of lysis inhibition [10 11] temporarily interrupts the lytic process thereby permitting the continuation of the reproductive process with an ultimate increase in burst size

The important work of Herriott[18] on the properties of the nucleic acid free ghosts of phage T2 presents further evidence for the independence of the two processes The phage ghosts are unable to initiate the reproductive process but retain the ability of lysing the host cell It might be said that the ghosts can produce abortive infection without being competent to initiate true infection The biochemical details of the reproductive and lytic processes are as yet unknown and present a major challenge for the future

Abortive infection has not been studied with animal viruses because techniques for investigating the fate of single infected animal cells have not been available However recently developed methods for the preparation of suspensions of isolated host cells and for the assay of virus infected animal cells by plaque counting methods will permit the study of the effects of the chemical and physical environment on virus development

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10

Metabolic Transformations in Virus Infected Cells*

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I shall confine my remarks to certain special metabolic problems of the T-even bacterial viruses The concentrated effort to understand these particular viruses which occurred in the past ten years was a most curious development in the history of virology That this effort was made can be considered to be a fortunate accident from the point of view of the development of the methodology of virology From the aspect of the general applicability of various results the study of the T-even phages has yielded many unique and disconcerting quirks especially at the level of metabolic behavior

One of the most striking of these quirks among the bacterial viruses proved to be the inability of infected bacteria to multiply and synthesize many normal host constituents [1] On closer examination there was revealed a shunting of phosphorus into the exclusive accumulation of virus deoxyribonucleic acid at the expense of the synthesis of the host nucleic acids [2] The parasitism displayed in these systems is most extreme leading to the almost exclusive synthesis of virus On further dissection this effect was revealed in the alteration of the balance of two major alternative paths of carbohydrate metabolism [3] All of these details of metabolism could be explained in terms of quantitative alterations of the utilization of the enzymes of the host to make the same old building blocks which were now incorporated in quantitatively different amounts into the specific polymers of virus

The discovery of a new pyrimidine 5-hydroxymethyl cytosine (HMC)

* This research was aided by a grant from the Commonwealth Fund

by Wyatt and myself[4] in virus nucleic acid which can not as yet be found in the host has raised new types of questions It is quite perplexing for instance that this compound has not been found anywhere other than in the T-even phages

The knowledge of the absence of cytosine and its replacement by HMC in virus nucleic acid has been extended by the finding that cytosine of host DNA may be converted to virus HMC in infected cells[5] As seen in Table 1

Table 1

BASES OF HOST AND VIRUS NUCLEIC ACIDS

E coli RNA	*E coli DNA*	*T2 T4 T6 Virus DNA*
Adenine	Adenine	Adenine
Guanine	Guanine	Guanine
Uracil	Thymine	Thymine
Cytosine	Cytosine	5-hydroxymethyl cytosine

cytosine is an essential constituent of both host nucleic acids RNA and DNA The compulsory synthesis of HMC in infected cells with the partial conversion of the host cytosine to the new base suggests that infected cells are unable to accumulate cytosine compounds and are hence unable to build structures containing host nucleic acids and contiguous compounds The synthesis of HMC rather than cytosine also suggests the basis for the exclusive accumulation of virus nucleic acid and its complementary elements of virus protein It is now proposed that the drastic reorganization of cell economy into virus synthesis is the result of a minute alteration of the qualitative character of a small building block cytosine The alterations of the balance of paths of carbohydrate metabolism and of the distribution of cell phosphorus as well as the inability to synthesize more cells are now suggested to be the secondary consequences of this critical reaction

What is the nature of this reaction? A scheme surveying some possible interrelationships of the pyrimidines is given in Figure 1 Weed and I have found that the hydroxymethyl group of HMC may be readily derived from the β carbon of serine[8] However the new pyrimidine and its deamination product hydroxymethyl uracil (HMU) so similar to thymine were found to be inert in filling the pyrimidine requirements of various pyrimidineless mutants of E coli[6] HMC is also inert to the cytosine deaminase of E coli and of yeast the enzyme of the latter being able to degrade 5-methyl cytosine to thymine[8] Further HMC is not utilized by growing or T2 infected cultures of E coli[7]

On the other hand the deoxyriboside of HMC is much more reactive Although the nucleoside does not fulfill pyrimidine requirements of our mutants it may be used as a source of deoxyribose by various organisms It is slowly deaminated by the very active deoxycytidine deaminase of E coli at a rate of 2 to 4% the rate on cytosine deoxyriboside The result

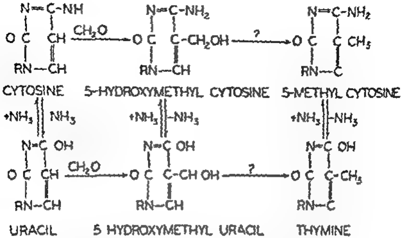

FIG 1 Some possible metabolic interrelations of the pyrimidines

ing HMU deoxyriboside although very similar to thymidine is inactive for pyrimidine requiring or deoxyriboside requiring organisms From these data I have tentatively concluded that the deoxyriboside of HMC is probably the compound which is metabolically active rather than free HMC Further it seems possible that a route for hydroxymethylation involves de aminated deoxycytidine with subsequent amination Finally the inertness of all the hydroxymethvlated nucleosides suggest that they win out in a competitive fashion over the normal nucleosides and that the hydroxy methyl derivatives are not normal intermediates in pyrimidine metabolism

In the course of studies on the isolation of the deoxyriboside of HMC it was also discovered that this compound is present in virus DNA in a linkage resistant to the combined action of deoxyribonuclease and alkaline phosphatase unlike the linkages which hold deoxycytidine in thymus DNA for example It may be suggested then that this linkage assists the irreversible trapping of HMC nucleotides in virus DNA and possibly assists the survival of virus DNA injected into infected cells

If the scales are so tipped in favor of HMC accumulation it is relevant to ask why hydroxymethylated compounds have not been found in normal metabolism Two unusual possibilities come to mind

(a) Essential portions of the hydroxymethylation mechanism i e enzyme or coenzyme are furnished by the virus or
(b) The infection evokes into activity a dormant enzyme system of the host

Of course other possibilities remain e g the insensitivity of our procedures for the detection of HMC or the special qualities of other virus compounds which may facilitate the trapping of HMC Nevertheless test of the first two hypotheses will probably permit us to learn some new bio chemistry and, these therefore seem most intriguing at the moment

The data which I have quickly summarized have been presented in some detail this Spring [5] At that time brief mention was made of a somewhat analogous finding with respect to thymine synthesis in virus infected cells and I wish to present this data of Barner and myself[6] in somewhat greater detail than was possible then

In order to test the ability of our hydroxymethylated derivatives to satisfy the pyrimidine requirements of appropriate mutants of E coli a cytosine or uracil requiring mutant of E coli strain W and a thymine requiring mutant of E coli strain 15 were obtained The growth responses of these organisms to various pyrimidines is given in Table 2 The thymine requiring

Table

COMPOUNDS PERMITTING GROWTH OF W _ AND 15_{T-}

Compound	*Strain W _*	*Strain 15_{T-}*
Uracil	+	—
Dihydrouracil	—	—
Orotic Acid	—	—
Uracil deoxyriboside	+	—
5–hydroxymethyl uracil (HMU)	—	—
HMU deoxyriboside	—	—
Cytosine	+	—
Cytosine deoxyriboside	+	—
HMC	—	—
HMC deoxyriboside	—	—
Thymine	—	+
Thymidine	—	+
5–methyl cytosine	—	—

mutant is seen to be quite specific It excretes a significant amount of a compound which permits the growth of the uracil requiring organism The thymineless organism designated 15_{T-} has many unusual properties It will support the multiplication of T2 but not of T4 T5 or T6 When in broth the organism will adsorb T2 virus although the adsorption rate con stant is considerably less than that obtained when strain B is used When grown in synthetic medium 15_{T-} adsorbs virus much less readily than when grown in broth In order to achieve infection readily for purposes of study in defined media it is necessary to grow organisms in broth wash and infect in synthetic media The medium used was a basic mineral me dium containing glucose and enriched by amino acids Under these con ditions cells infected in the presence or absence of thymine produce virus at 37 with a latent period of 33 minutes and a small burst size of 10 to 16

Since it is known that host DNA may contribute thymine for virus syn thesis it appeared desirable to see whether a net synthesis of DNA had taken place In the absence of thymine in the medium the uninfected organism is unable to synthesize significant amounts of DNA In addition 90% of the cells appear to die per hour i e can not be counted by a plate counting technique As shown in Figure 2 in the absence of thymine the

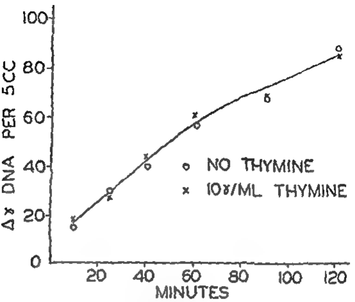

FIG 2 The synthesis of DNA in 15_{T-} infected by T2r+ in the presence or absence of thymine

cell makes virus DNA at a rate as great as if thymine were present in the medium Even when the viable count is down to 5% of the initial count as a result of holding the cells in the absence of thymine on infection the cells are still able to synthesize virus DNA at much the same rate

An interesting observation has been made concerning the death of cells in the absence of thymine It has been found that death may be prevented by substances other than thymine which do not support cell multiplication Such a substance is present in broth from which thymine has been exhausted A comparable substance is also excreted into the synthetic medium by cells grown in the presence of thymine The results seem to suggest a metabolic role for thymine in the production of an essential metabolite and the nature of this relationship is being investigated

The virus produced by the cells in the absence of thymine is T2r+ according to the usual biological criteria It has been isolated and its base content has been determined as presented in Table 3 The preparation of virus obtained under these conditions contained more thymine than was originally present in the uninfected cells

As can be seen in Table 3 the bases synthesized in infected cells in the absence of thymine had a molar composition fairly close to that of virus Furthermore these bases included the new pyrimidine HMC as well as thymine The net synthesis of purines and pyrimidines in 15_{T-} infected in the absence of thymine in the medium can be seen in Figure 3 and it may be noted that thymine synthesis is the most extensive of any of the bases

Table 3

SYNTHESIS OF BASES IN THYMINELESS E COLI (15_{T-}) INFECTED BY T R+

System	*Adenine*	*Guanine*	*HMC*	*Thymine*
T2r+ from B	3 0	18 0	16 8	33 3
T2r+ from 15_{T-} A With added thymine	79 9	17 6	16 8	35 7
B Without added thymine	30 8	17 7	16 5	35 1
Infected cells (15_{T-} — T2) 2 hours	33 5	16 7	13 0	36 8

Indeed in this system we have some evidence that thymine is even excreted to a slight extent It appears therefore that T2 infection can induce the synthesis of two pyrimidines which the uninfected cell appeared incapable of making In the special case of 15_{T-} the newly evoked synthetic functions account for all the accumulated pyrimidines

One problem which arises is similar to that described in the discussion of the significance of HMC Is the uninfected cell really incapable of making

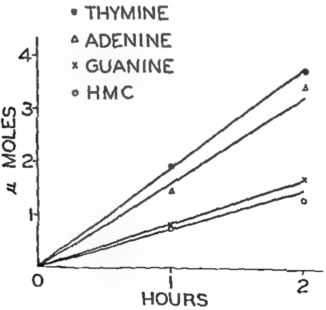

FIG 3 The synthesis of purine and pyrimidine bases in E coli 15_{T-} infected by T2r+ in the absence of thymine in the medium Each point represents net synthesis in 300 ml of culture

thymine? We find that thymine accumulated in host DNA during growth is never in excess of that removed from the medium We are preparing to test more critically for a small amount of thymine synthesis in the presence of thymine in the medium Such a finding would, of course suggest more strongly the unmasking of enzymes present in the cell

The phenomena described in this paper are the first to suggest that a virus may supply some important enzymatic element essential to the bio synthesis of virus It is recognized that the evidence for this is indirect and that alternative hypotheses such as those presented in this paper have equal force at present On the other hand the case may perhaps be analogous to the induction of toxin formation by the infection of avirulent C diptheriae and the problems raised by that finding That phenomenon is more reason ably explained (although not exclusively) as a disturbance of the metabolism of the host cell than are the phenomena of transduction and the acquisition of metabolic function which occur in other lysogenic systems The latter seem to have their basis in the transmission of unique genetic elements The phenomena in E coli and C diphtheriae do seem to be more susceptible to biochemical study and it may be that the answers to these problems in these organisms can be given in the not too distant future in chemical terms

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11

Intracellular Sites Important to the Development of Animal Viruses*

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It is only in conjunction with some host-cell that the special circumstances are met in which the maximum potential of the viral mechanism is evident Under these very exacting conditions viral replication is accomplished Even at our present level of understanding it can be said with certitude that the cell supplements the metabolic activities in which the elementary body is so clearly deficient Many of these represent coupled synthetic and exergonic reactions for which molecular elements may not be easily substituted [1] However it is not clear whether the essentiality of the environment can be accounted for wholly in terms of its biochemical properties The question remains does the physical state or any cellular apparatus have a role in the replication process?

Recently developed techniques have been employed to relate the functions of intracellular structures one to another [2,3] The synthesis of many small molecules has been found to be localized intracellularly Some of these are important in the synthesis of large molecules of the cell and hence are also necessary for viral replication Such relationships between virus and cell which are several times removed are easily found Our concern must be whether any intracellular structures are important to viral development in some manner over and above that necessary to normal cellular function More precisely is there a step unique in the viral development which requires the physical environment of the host Such a step might be the polymerization of nucleotides or amino acids or even the formation of unique precursors not found in the final viral body In the broadest biochemical

Many of the studies reported in this paper were aided by a grant from the National Foundation for Infantile Paralysis

sense, the latter might include any special adaptive enzymes In these terms it may be gainful to ask where within the cell is the virus formed

In the normal cell localization of chemical reactions is achieved by a combination of low operating temperature and the use of enzymes The function of the enzyme in this capacity is undoubtedly one of the most wide spread evolutionary developments in biology If the formation of virus is localized in the cell it is probably because the determinants of replication the nucleic acid or other catalysts are attached to some intracellular structure It suffices to say that little is known of the role of cellular organization in the synthesis of normal proteins and nucleic acid and for some time the study of viruses may be expected to contribute more to this problem than the converse approach

For the investigator the animal cell is particularly suitable material The supporting background of cytological information as well as size are favorable factors Furthermore these cells can be fractionated in mass by techniques which yield fragments of morphological significance Such techniques have been applied to tissues infected with herpes and poliomyelitis viruses [4 5 6] While admittedly evidence concerning immature forms would be of greater significance by necessity these studies have concerned only mature fully infectious virus

Infection with herpes simplex or poliomyelitis viruses produces significant nuclear aberrations in the host cell In the latter case a dissolution of the Nissl substance is seen and in the former an inclusion body While vaccinia and psittacosis viruses produce cytoplasmic inclusion bodies which undoubtedly represent developmental forms no such clear interpretation can be made regarding these nuclear aberrations It was from the viewpoint of investigating these nuclear changes that the isolation of cellular structures from infected tissue was first undertaken

Cellular fractions corresponding to nuclei mitochondria and supernatant fluid were prepared from embryonic chick liver which was heavily infected with herpes virus The method of Schneider and Hogeboom was employed in which the tissue is disrupted in a non ionic medium (25 M sucrose) [7] The results obtained when these fractions were titered for herpes virus are shown in Table I The nuclei mitochondria and supernatant fluid contained correspondingly 1 3 16 and 80 per cent of the virus found in the whole homogenate [8] Clearly the major part of the virus is in the supernatant fluid which contains the soluble proteins and a collection of diverse small particulates termed microsomes It should be recalled that the nuclear fraction contains a few whole cells blood elements and some contaminating mitochondria The low viral activity of the nuclear fraction may be accounted for in terms of these impurities

When tissues of the central nervous system obtained from cotton rats infected with poliomyelitis virus were fractionated by the same technique Schwerdt and Pardee found an almost identical distribution of poliomyelitis

Table I

INTRACELLULAR DISTRIBUTION OF HERPES VIRUS

Exp	Virus Titers of Cellular Fractions			
	Whole Homogenate	*Nuclei*	*Mitochondria*	*Supernatant Fluid and Microsomes*
I	7.0	4.8	—	—
II	6.5	4.5	—	—
III	6.5	—	6.0	—
IV	6.3	—	5.6	—
V	6.8	—	—	6.5
VI	6.4	—	—	6.4
Average	6.6	4.7	5.8	6.5
Percentage	100	1.3	16	80

The 50% infectious dose for eggs is expressed as the reciprocal of the log of the dilution of the cell fraction obtained from 100 mg of liver

virus These workers also believed that the virus of the nuclear fraction was accountable in terms of the cytoplasmic contamination of that fraction [6]

On the basis of present information it cannot be proved that herpes virus is not released from nuclei before or when the cells are disrupted by the procedures of purification employed However the high count of nuclei in preparations of infected liver indicates that if virus is released from these bodies there is not sufficient concurrent destruction of them to account for the low concentration of virus in that cellular fraction Infected livers contain a slightly but consistently greater number of nuclei per unit weight than normal tissue Embryonic liver from the chick at fourteen days of development contains 76×10^7 nuclei per gram of moist tissue [18] Further the intracellular distribution of desoxyribonucleic acid of infected tissue shows there is no unnatural amount of this nuclear constituent in the cytoplasmic fractions as might be expected if the nuclei of infected cells were unusually fragile [9]

When tissues are disrupted in isotonic saline an insoluble residue composed of agglutinated particulates of the nuclei and cytoplasm is obtained After exhaustive extraction with 0.85 per cent saline the available virus in this residue can be markedly increased by treatment with M saline The latter reagent is known to cause nuclear disruption This phenomenon was first reported by Francis and Kurtz who were working with herpetic chick liver [4] However a residue with these properties can also be prepared from mouse brain which is infected with poliomyelitis virus (Kaplan and Melnick)[10] or with Theiler s virus (Gard and Ostlund) [11]

Kaplan and Melnick have attributed significance to the traces of virus found in the nuclear fraction and interpreted the effects of M saline in terms

of a hypothesis of nuclear localization of poliomyelitis virus The inadvisability of these interpretations becomes clear from consideration of several additional pieces of evidence presented largely by Gard and Ostlund Firstly, residues with these properties can be prepared from normal mouse brain and the cytoplasm of infected tissue Secondly the action of M saline is not unique and the bound virus can be released by techniques which should not disrupt the nuclei [11] Thirdly the remaining residue which is insoluble in M saline and is presumably cytoplasmic material contains most of the virus newly made available [4]

If the conclusion is to be reached that the nucleus is the site of viral replication in the case of herpes or poliomyelitis viruses it must not be on the basis of the data presently available which deal with intracellular localization of infectious virus There still remains the problem of the significance and nature of the nuclear aberrations seen in cells infected with these viruses In this regard it is of interest to note that nuclei isolated from herpetic livers contain the same amount and ratio of RNA to DNA found in normal liver The respective normal values being $4\,2 \times 10^{-7}$ and $2\,2 \times 10^{-7}$ μg per nucleus Further the entire intracellular distribution and amount of nucleic acid of the liver is unaltered to any detectable degree under the circumstances of herpes infection [9]

The major part of the intracellular virus (80 per cent) was found in the cytoplasm in what appears to be a form uncombined with nuclei or mitochondria This finding holds for infection both with herpes and poliomyelitis viruses Schwerdt and Pardee also subfractionated the microsomes of nervous tissue and concluded that most of the virus is in a free state and not adsorbed to the submicroscopic particulates [6]

In addition to the data from cellular fractionation there is considerable evidence that relates the mitochondria to the development of virus Firstly with influenza virus there is a metabolic relationship Under the influence of certain enzymic inhibitors the respiration of the host tissue an activity associated with the mitochondria can be depressed and under these circumstances the yield of virus produced is directly proportional to the residual oxygen consumption [12] This is illustrated in Figure 1 where the effects of antimycin–A on chorio allantoic membrane infected with influenza virus are shown Secondly there is an alteration in the pattern of enzymes which are known to be localized in the mitochondria Under the influence of herpes infection there is a decrease in succinoxidase and α–ketoglutaric acid oxidase which is particularly marked in embryonic heart tissue [8] Further it has been reported by Schwerdt and Pardee that infection with poliomyelitis virus lowers the succinoxidase activity of the central nervous system [6] Thirdly there is evidence that mitochondria of liver tissue undergo a selective deterioration when infected with herpes virus From Table 2 it will be noted that there is a 30 per cent decrease in the mitochondrial nitrogen per unit weight of infected liver as well as a 36 per

RELATIONSHIPS OF ANTIMYCIN-A CONCENTRATION-
OXYGEN UPTAKE-AND VIRUS PROPAGATION

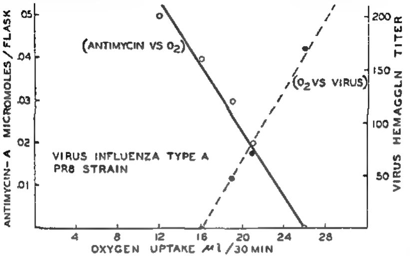

FIG 1

cent decrease in the mitochondrial nitrogen per nucleus These data indicating a selective destruction or deterioration of mitochondria are supported in part by electron microscopy [13]

Since the mitochondria had a considerable viral titer and are the only particulates which were associated with a significant amount (16 per cent) of the total virus of the cell an attempt was made to determine whether

Table 2

EFFECT OF HERPES INFECTION ON THE AMOUNT OF MITOCHONDRIA PER CELL

Exp *	*Tissue*	*Nuclei/gm Liver* $\times 10^7$	*Mitochondrial Nitrogen* mg/gm Liver	*Mitochondrial Nitrogen* µg/Nuclei $\times 10$
I	Normal	50 9	5 76	11 3
	Infected	54 3	4 68	8 6
II	Normal	7?	6 88	9 5
	Infected	80 0	4 36	5 5
III	Normal	73 0	5 15	7 1
	Infected	84 0	3 45	4 1
Average	Normal	65 4	5 92	9 1
	Infected	7 8	4 19	5 8

In each experiment a pool of livers obtained from 18 eggs was used

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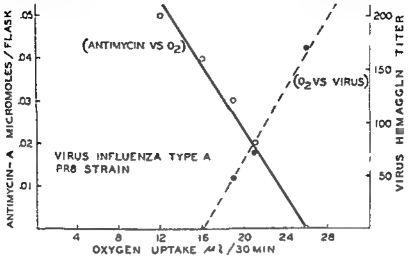

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III	Normal	73 0	5 15	7 1
	Infected	84 0	3 45	4 1
Average	Normal	65 4	5 9?	9 1
	Infected	72 8	4 19	5 8

In each experiment a pool of livers obtained from [illegible] eggs was used

the viral activity was a superficial contamination To do this the sedimentation characteristics of the virus under these particular conditions were determined Both nuclei and mitochondria can be sedimented from a homogenate by a centrifugal force of 2400 $\times$ G applied for twenty minutes It is seen from Figure 2 that repeated centrifugation with this force did not remove the virus from the supernatant fluid However if one repeats the operation employing a force of 24 000 $\times$ G the virus is readily sedimented

Mitochondria obtained from liver infected with herpes virus were washed repeatedly in cold isotonic sucrose After each washing the residue was sedimented under conditions known not to sediment the virus Titrations of the mitochondria after each of five successive washings revealed a constant titer by the second washing This demonstrates that the virus was being repeatedly sedimented with the mitochondria Thus a considerable amount of virus was associated with the mitochondria in what was apparently an intimate attachment It is of interest to note that the virus bound to these particulates is still available to the action of immune serum

In contrast to the above results when a reconstituted mixture composed of mitochondria from normal tissue and supernatant fluid from a homogenate of infected tissue was subjected to the same procedure the mitochondria and virus were readily separated Figure 3 Thus under the conditions employed to isolate infected mitochondria there was no signifi

EFFECT OF REPEATED SEDIMENTATIONS ON THE VIRAL CONTENT OF THE SUPERNATANT FLUID

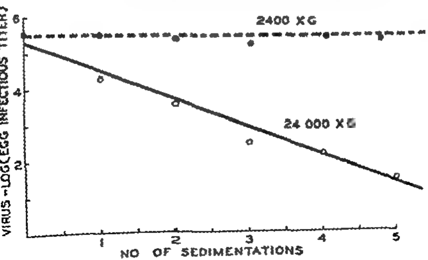

FIG 2

cant union of virus and normal mitochondria In addition attempts to achieve union in the presence of ionic medium or at 37 C were also without success

The story regarding the relation of poliomyelitis virus to mitochondria of nervous tissue is remarkably similar The specific activity of a whole homogenate of infected brain was $10^{-5.4}$ while that of the mitochondrial fraction was $10^{-4.9}$ Even after four successive washings[6] the titer was still 10^{-4}

EFFECT OF REPEATED WASHINGS ON THE VIRAL CONTENT OF MITOCHONDRIA

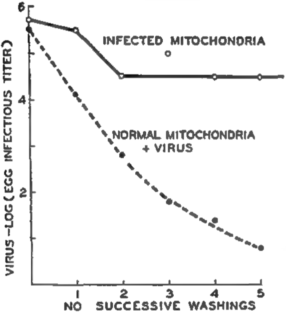

FIG 3

Possibly the loss of mitochondrial material just as the nuclear aberrations could be secondary to viral injury at some other site A similar decrease in mitochondrial nitrogen has been recently described in connection with adaptive enzyme formation in the liver of the rat [14] However in view of the intimate attachment as well as biochemical relation of herpes virus to the mitochondria it was tentatively advanced that these organelles were an intracellular site of viral synthesis Later deterioration of the mitochondria on maturation of virus would account for the accumulation of large amounts of free virus in the cytoplasm

While it might be rewarding to extend these observations with a direct visualization of herpes virus in isolated cellular structures it is unlikely that the significance of any particle so visualized can be established until the morphology of the virus is known Already particulate materials interpreted as virus have been found in a number of sources of herpes infection Unfortunately these vary in diameter from 90 to 233 mμ [1 ... 17] Our own estimates[19] made upon preparations which we know to be impure are in the range of 96 to 116 and these are in agreement with values submitted by Reagan et al [17]

Perhaps more appropriate at this time than electron microscopy would be the study of intracellular distribution of herpes virus as a function of time However for this the techniques using intact animals seem completely inadequate The first herpes virus to appear in the organs of the chick embryo undoubtedly will be found in the blood which carries the virus from the extra-embryonic membranes Since some blood elements are always found in the nuclear fraction it will be difficult to determine whether the earliest virus detected there particularly if it is small in amount is relevant to viral development in the hepatic cell To circumvent these

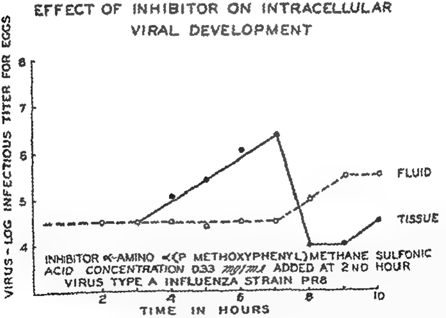

FIG 4 Each culture contained 400 mg of tissue The inoculum was added at zero time and consisted of 0 3 ml of medium with a titer of 10^{-4} One culture was used to obtain data for the points at each time interval The tissues were ground separately with alundum and the titers obtained represent the infectious titer expressed per gram of tissue The titers of the fluids are recorded on a per ml basis

difficulties it will be necessary to use a pure culture of cells in which all cells can be infected simultaneously In many respects the Hela strain of human tumor cells grown in tissue culture might be the most workable material[19]

Since the virus of animal cells is produced and released over a consider able period of time even this approach may not yield unequivocal results If the virus could be blocked from leaving the cell or from leaving the site of development clear results might be obtained The most interesting ma terial in this regard is the viral inhibitor α-amino-α-(p-methoxyphenyl) methane sulfonic acid[20, 1] A process occurs early in the development of influenza virus which is sensitive to the action of this compound If the in hibitor is added after this process is completed i e thirty minutes after the virus inoculum replication of virus will occur but the release of virus from the chorioallantoic membrane is impaired[21] The apparent latent period as measured by the release of virus into the extra-cellular fluid may be ex tended as much as three times (Figure 4) Where the virus is located in the cells under these conditions of liberation inhibition may be a question of some pertinence The proper use of metabolic antagonists particularly in conjunction with some of the newly available techniques for manipulating animal viruses can be expected to yield an ever increasing amount of sig nificant biochemical data not only relevant to intracellular sites important to viral development but also to problems concerning the external membrane

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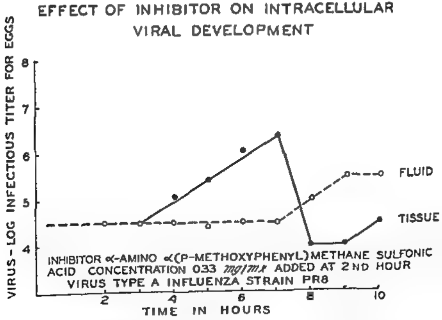

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12

Enzymic Changes in Virus Synthesis*

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Department of Biochemistry University of Chicago Chicago Illinois

Studies of the enzymic changes produced by animal viruses in susceptible host cells have revealed a variety of changes and with the advent of tissue culture methods which may permit the large scale cultivation of animal viruses one can hope for a great increase in such information In the time at my disposal however I shall confine myself to the limited and special case of the bacteriophage system Dr Cohen has already discussed several aspects of this subject and certainly the phenomena that he describes are of great importance in our understanding of viral reproduction

It is clear that infection of a bacterial cell by a bacteriophage particle is not necessarily followed by the classical picture involving viral reproduction and eventual lysis of the host One can distinguish at least two and possibly three kinds of phenomena subsequent to phage infection

First with the so-called temperate phages invasion of the bacterial cell by the virus *involves unknown reactions which lead to the transformation* of the phage into the so-called prophage The host cell continues to grow and reproduce in a normal fashion with the prophage also being reproduced as an essential component of each daughter cell originating from the parent Whether the prophage is a specific molecular entity or whether it is some persisting alteration in the normal metabolic apparatus of the bacterial cell is uncertain although there is evidence that the prophage is closely associated with the genetic material of the cell Cultures of bacteria containing prophage are termed lysogenic i e that in any growing culture of such cells one finds that in a small proportion of the cells the prophage is spontaneously transformed into an active vegetative particle which proceeds then to develop into mature phage This results in the lysis of the particular cells concerned with the liberation of phage particles into the medium This

* Aided by grants from The National Foundation for Infantile Paralysis Inc and the D Wallace C and Clara A Abbott Memorial Fund of the University of Chicago

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was demonstrated It is possible that under these circumstances the phage particles may be regarded as passive carriers of single hereditary traits although the exact nature of the phenomenon involved is not clear

Our information as to the enzymic changes undergone by the host cell during these various types of infection is still very meager We know most of the events involved in viral replication in the case of the virulent phages

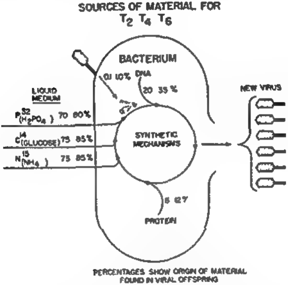

FIG 2

Figure 2 is a summary of experiments dealing with the origin of the various components of the viral progeny in the case of the even numbered T phages T2 T4 and T6 which attack the *E coli* strain B Since one infecting particle can lead to the formation of as many as a thousand identical offspring it is clear that a net synthesis of considerable magnitude is occurring and it is possible by the use of isotopic tracers to demonstrate the origin of the various components of virus progeny As you see 70–80% of the actual substance of the viral offspring is synthesized from the ammonia and glucose and phosphate of the medium A small amount of the protein of the virus comes from the protein of the bacterial cell In other words then synthesis of these phages involve the utilization of small amounts of bacterial protein and practically all of the DNA of the bacterial cell accompanied by synthetic reactions of large magnitude in which viral DNA and protein are synthesized directly from the components of the medium It is also clear that the large scale utilization of bacterial DNA does not involve the transfer of large fragments of this material but as one

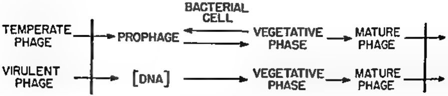

FIG 1

occurs spontaneously to a small extent and for unknown reasons But it is also possible to cause a practically complete conversion of the prophage into the vegetative state by treating the prophage containing cells with a variety of so called inducing agents These include ultraviolet light X rays sulfhydryl compounds certain mutagenic and carcinogenic agents etc When such substances are applied under the proper conditions to the cell containing prophage conversion of the prophage to the vegetative state occurs and one observes practically completely lysis of the culture with the liberation of large amounts of virus particles The phage produced under these circumstances is identical with the original infecting particle and when absorbed in the same host will undergo again the series of changes I have outlined

All of this is in marked contrast to the behavior of the so called virulent phages When virulent viral particles are adsorbed by a sensitive host the infecting particle is fragmented or split and it appears that only the nucleic acid of the virus (i e its DNA) participates in the further stages of virus reproduction although a small amount of parent protein may also be in volved In any event it is impossible to demonstrate for a time the presence of mature virus particles inside the cell but shortly they appear in increas ing numbers until the cell lyses with the liberation of as many as a thousand particles identical with the original parent

In the third type of bacterial invasion by bacteriophage the role of the bacteriophage particle is not entirely clear but it is possible that it is in volved in the transmission of single hereditary traits Zinder and Lederberg[1] found that two different auxotrophic mutants of Salmonella typhimurium when separated by means of a ground glass filter exchanged genetic char acteristics by way of a filterable agent designated FA They partially characterized the FA by centrifugation electron microscopy and diffusion through membranes In size appearance and activity FA was found to bear a close resemblance to the lysogenic phage of Salmonella typhimurium More recent experiments by Baron and his collaborators have involved a mutant form of Salmonella typhosa Ty 2 characterized by its ability to ferment zylose and by its resistance to streptomycin This strain was lysed with phage V 1 and the lysate purified A receptive strain of Salmonella typhosa which was xylose negative and streptomycin sensitive was then incubated with the purified lysate Significant reversion to the original host

was demonstrated It is possible that under these circumstances the phage particles may be regarded as passive carriers of single hereditary traits although the exact nature of the phenomenon involved is not clear

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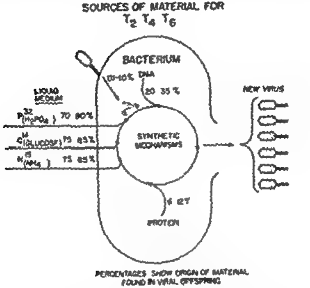

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can show by tracer experiments proceeds by the preliminary breakdown of DNA into smaller fragments and the utilization of these for the synthesis of virus DNA However even though there appears to be a preferential utilization of host DNA it seems that there is nothing obligate about this transfer since the viru particles synthesized in the latter stages of the reproductive process contain relatively smaller quantities of host materials

In addition to these transfers a portion of the substance of the infecting particle also appears in the viral offspring but this is small in amount and there is no evidence that the transfer of this material is an obligate feature of viral synthesis The picture emerging from these studies indicates that with the virulent phage the infecting particle loses its physical integrity in the early stages of the process and that while there is utilization of the actual material of the parent virus and possibly preferential utilization of portions of the bacterial host cell there is no evidence that these transfers are necessary for viral synthesis to proceed

It follows that enzymatic mechanisms for these various changes must be present in the infected cell and that such mechanisms must be derived either from existing components or by *de novo* synthesis However it is not certain that all of the changes observed in infected cells are necessarily involved in virus synthesis

In the case of such virulent phages as those of the T group acting on *E coli* strain B infection is followed by cessation of bacterial growth If the oxygen uptake of the infected bacteria is measured it is found that respiration continues but that the increase found in growing cells is not observed That is the bacteria respire at the rate existing at the time of infection and it appears therefore that there is no increased synthesis of the enzymes involved in the respiratory mechanism The synthesis of RNA is also blocked with infection and the quantity of RNA present remains the same throughout the latent period The infected cell is incapable of forming adaptive enzymes Any adaptation taking place at the moment of infection is immediately stopped and the amount of adaptive enzyme present in the cell remains unchanged After a short latent period one can observe increases in the amount of both protein and DNA present and these latter processes continue until the cell is lysed

However some of these changes can be demonstrated in the absence of actual viral reproduction Herriott and his collaborators[3] have studied properties of the ghost forms formed by osmotic shock treatment of the phage T2 In bacterial cells treated with an average of three ghosts per cell they found RNA synthesis blocked but DNA and protein were formed in appreciable quantities So far as I am aware direct examination of the chemical nature of the DNA synthesized under these circumstances has not been made but Herriott suggests on the following grounds that the DNA synthesized under these circumstances is probably not that characteristic of the virus Treatment of the normal host cell with dilute aqueous sulfur

mustard was found to block the synthesis of DNA These same cells how ever were able to produce normal quantities of T2 virus which meant that they were able to synthesize virus DNA although unable to form host DNA Herriott and his associates now find that host cells treated in the same manner with sulfur mustard and then infected with the ghosts of T2 were not able to synthesize DNA although some protein was formed This would also suggest that the material injected by the virus which appears to be primarily DNA is needed for viral DNA synthesis—perhaps as Herriott suggests—to replace a key component of the synthetic mechanism of the host destroyed by treatment with the sulfur mustard

The changes observed after infection by a temperate phage differ markedly from those already described After a prophage carrying bacterial cell has been treated with an inducing agent for example ultraviolet light the oxygen uptake of the cell continues to increase and the cell continues to grow although cell division does not occur These cells continue to synthesize RNA and are capable of forming adaptive enzymes almost up to the time mature virus particles can be detected in the cell One also observes the increased synthesis of DNA and protein which is the common feature of both types of infection

It is not clear why with the virulent phages the inhibition of the synthetic reactions of the infected cell should be so much more extensive Jacob[4] has made an interesting study of this phenomenon comparing the infection of *P pyocyanea* with the virulent phage P2 to infection with the temperate phage P8 In his experiments glucose served as the sole carbon source for the bacteria and could be introduced at a controlled rate When the available glucose concentration was reduced beyond a certain point then bacterial growth was retarded However in bacteria infected by the virulent phage Jacob found it possible to reduce the amount of available glucose to ⅓ or ¼ of that necessary for growth without affecting the yield of virus per bacterial cell It was only after the environment contained less than ⅕ of the carbon necessary for bacterial growth that the yield of phage particles per cell declined

In *P pyocyanea* infected with the temperate phage P8 and induced with ultraviolet light he found again that phage synthesis is the last process to be affected by a decrease in the concentration of available glucose As the glucose concentration of the medium is lowered RNA synthesis and the ability of the cell to form adaptive enzymes are all decreased However the phage yield remained unchanged and it was necessary to lower the concentration of glucose available to ⅓ or ¼ of that required for growth before the synthesis of virus was decreased Two conclusions derive from these experiments first that in spite of the synthetic limitations that occur with the virulent phages apparently a considerable portion of the metabolic activity of the infected cell is not directed to virus synthesis and secondly it would appear that in the case of both virulent and temperate phages phage

synthesis has a preferential claim on the energy and material sources available to the infected cell

I have already mentioned the fact that infection with the virulent phages does not cause any increased synthesis of the enzymes associated with the respiratory system and it is of interest to inquire whether all the energy available for viral synthesis is used directly for that purpose or whether the cell must use part of the available energy to form new enzymes and other metabolic machinery Evidence bearing on this point is still incomplete

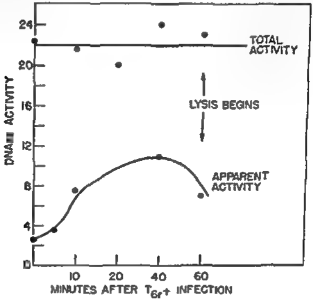

FIG 3

In the case of the increased synthesis of the pentose desoxyribose which must occur in the infected cell Cohen has shown that this is affected by a quantitative shift in the balance between enzymic reactions already existing in the normal cell

Both Pardee[5] and Kozloff[6] have observed a striking increase in the DNAase activity of infected cells although the exact physiological role of this enzyme in virus synthesis is not clear Kozloff in our laboratory has shown that the two or three fold increase in the DNAase activity of infected *E coli* cells can be ascribed primarily to a decrease in the presence of a naturally occurring inhibitor *In the uninfected cell the inhibitor is present* in excess and little or no DNAase activity can be demonstrated in normal cells As infection proceeds the concentration of this inhibitor decreases although some still remains at the moment of lysis Dr Kozloff at the moment is involved in studying the chemical nature of both the DNA and the inhibitor He has purified the enzyme some 60-fold The inhibitor

proves [redacted] be heat stable nondialyzable unaffected by shaking with chloroform and [redacted] apparently a specific ribose nucleic acid component of the bacterial cell As one would expect the inhibitor can be destroyed by ribonuclease Centrifugal fractionation of sonic extracts of *E coli* show that the inhibitory RNA comprises a small fraction of the total RNA in the cell The action of the inhibitor is quite specific inasmuch as DNAase from pancreas is unaffected by it The inhibitor apparently combines directly with the enzyme since the reaction between inhibitor and enzyme which occurs instantaneously is unaffected by the source of desoxynucleic acid used as a substrate and can be reversed by treating the inhibited DNAase with ribonuclease

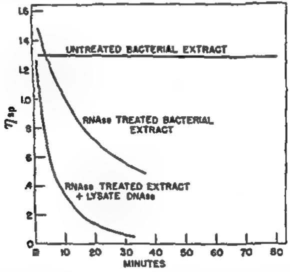

Fig 4

At the moment data supporting the view that new enzyme systems are created during viral replication is derived entirely from the experiments concerning hydroxy methyl cytosine (and thymine?) already described by Dr Cohen Insofar as DNAase activity is concerned [redacted] with the carbohydrate synthesis an alteration in known enzymic processes occurs rather than the appearance of [redacted] qualitatively new reaction

However we are merely at the beginning of an exploration of the enzyme systems involved in virus synthesis and the data available are still insufficient to permit general conclusions

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DISCUSSION

Mechanisms of Virus and Rickettsial Infections

DR HORSFALL In regard to Dr Commoner s report particularly the immunological data I wondered whether he had attempted to use cross absorption techniques with a view to determining whether he was dealing with a mixture of antibodies or specific antibodies directed only against TMV The reason for this question is some concern regarding the possible contamination of the B3, B6 and A4 components with small amounts of TMV or vice versa

DR COMMONER We have used the agar diffusion technique to confirm the cross reactions among TMV and the nonvirus proteins These tests show that the reactions of TMV with anti B8 serum and of B8 with anti TMV serum represent single component systems Furthermore from diffusion rates it can be shown that in a given serum the same antibody reacts with the homologous and heterologous antigen

DR KIDD (Moderator) Dr Commoner while you still have the microphone would you be willing to consider a more general question? What would be the simplest explanation of the significance of the nonvirus proteins?

DR COMMONER I am very reluctant to propose such an explanation We still know so little about protein synthesis in general and about the biochemistry of TMV reduplication in particular that there is no justification for making the assumptions which would be required by any effort to fit our data into a simple explanation It is better I think to admit our ignorance and to work for more data

DR WOOLLEY I almost hesitate to come up here in view of Dr Commoner s remarks but then I feel that progress is only made by putting two and two together to make four so I want to try and do it This is in connection with Dr Cohen s most interesting findings Let us first remember what is now considered to be the mechanism of formation of thymine and of methionine The methyl group of methionine is thought to arise first as an hydroxymethyl group (attached to the sulfur of hemocysteine) which is then reduced to $-CH_3$ The carbon of this methyl group comes from

formatic and related compounds which constitute the so-called pool of one carbon compounds Similarly it has been shown that the methyl group of thymine comes from this one-carbon pool We can picture the reactions starting from let us say cytosine as the formation of 5–hydroxymethyl–cytosine which is then reduced to 5–methyl–cytosine This latter compound is a known constituent of some desoxynucleic acids and could of course yield thymine by simple deamination The reaction sequence here therefore would be quite analogous to the one believed to function in the formation of methionine Now let us apply this idea to Dr Cohen s findings He has observed the accumulation of 5–hydroxymethyl–cytosine in the phage What I wish to suggest is that the phage constitutes a specific block in the normal synthesis of methylcytosine and/or of thymine Since the normal process is thus blocked the 5–hydroxymethyl–cytosine accumulates and is incorporated by the virus into its nucleic acid The phage infection thus results in a specific block at this particular point

DR LURIA Dr Commoner has stated that the problem of the synthesis of the tobacco mosaic virus is a problem in altered protein metabolism In the light of what is known for bacteriophages it would seem logical to suppose that the primary problem may be one of altered metabolism of nucleic acid and that the metabolism of proteins is altered in a secondary fashion In connection with Dr Ackermann s paper I think that it is perfectly correct to say that herpes virus *is found* in the mitochondrial or microsomal fraction but as long as measurements are limited to infectious virus we are not justified in interpreting the results in terms of the site of synthesis of herpes virus

DR COMMONER In answer to Dr Luria s point I should like to agree that TMV reduplication is a problem of *nucleoprotein* synthesis rather than the synthesis of protein alone There is of course no justification for restricting one s approach merely to protein synthesis The virus is itself a nucleoprotein and there is considerable evidence that nucleic acids are intimately involved in all protein synthesis

DR L DMOCHOWSKI (University of Leeds England) There is one question I should like to ask Dr Ackermann in connection with his interesting studies on fractionation of virus of herpes If I have understood him correctly he has mentioned that the infective property of herpes is in part associated with the mitochondrial fraction of infected cells Now I for one am hesitant at the moment to accept this and if comparison is possible between one viral agent and another I would like to mention experiments carried out in collaboration with my associates on fractionation of spontaneous mammary tumours in mice which have the so called mammary tumour inducing agent or virus In the fractionation studies we carried out

in an attempt to ascertain the distribution of the mammary tumour agent among the various cellular constituents we obtained results similar to those mentioned by Dr Ackermann The various fractions of tumour cells obtained by differential centrifugation were serially diluted and injected into suitable test mice Serial dilutions of all fractions were made because it was felt that only in this way could a true picture of the distribution of the agent be obtained when combined with appropriate electron microscope investigations of these fractions It was found that in dilution of 10^{-6} the homogenate that is the fraction obtained after removal of unbroken cells debris and nuclei the incidence of tumours in the test mice was 90% nuclei in the same dilution gave only a 10% incidence of tumours in the test mice while mitochondria were inactive and in a lower dilution that is in a dilution of 10^{-} mitochondria gave an incidence of 20% of tumours in the test mice the so called large microsomal fraction induced a tumour incidence of 30% in a dilution of 10^{-6} and an incidence of 50% in a dilution of 10^{-5} finally the so called small microsomal fraction gave an incidence of 50% of tumours in a dilution of 10^{-6} and 70% incidence in a dilution of 10^{-5}

The application of electron microscopy has shown that the nuclear and mitochondrial fractions for some unknown reason contain a considerable number of microsomes and in spite of repeated washings the microsomes are still in these fractions—perhaps in diminished numbers but still there

We came to the conclusion therefore that the tumour inducing activity of the nuclear and mitochondrial fractions as far as the mammary tumour agent was concerned was due to the presence of small microsomes in the fractions that is particles of an average diameter of 300 A The so-called large microsomal fraction of tumour cells was composed of particles which appeared to be aggregates of small microsomes and similar in appearance to the loganberry fruit as well as free small microsomes and hence the comparatively high biological activity of these fractions

From the results of the biological tests and the electron microscope studies we concluded therefore that the tumour inducing activity of the mammary tumour agent is present mostly in small microsomal fractions and such activity as is associated with either the nuclear or mitochondrial fractions is only the outcome of contamination of these two fractions with small *microsomes*

I would like to hear Dr Ackermann s views on the above comparison of his results with ours and also whether in his studies he has carried out any electron microscopy

DR ACKERMANN We know little of the relation of the virus to the microsomes however the virus we find associated with the mitochondria of infected tissue does not seem to result from contaminating microsomes If one mixes mitochondria obtained from normal tissue with a supernatant fluid prepared from infected tissue it is possible to separate the virus from

the mitochondria However I doubt if the reisolated mitochondria are entirely free of microsomes We have not attempted electronmicroscopy because we doubt whether the morphology of the herpes virus was sufficiently well established that one could recognize the elementary bodies in tissue preparations if they were encountered

DR T E CARTWRIGHT (University of Pittsburgh) In view of Dr Adams remarks I should like to hear Dr Commoner s comments on some of the work which appears in the literature concerning the apparent multiplication of tobacco mosaic virus at the expense of the normal nucleoprotein

DR COMMONER This question refers to the work of Wildman *et al* who reported that TMV was synthesized by conversion of pre-existing normal protein This proposal does not conform with the facts The reasons for this conclusion are the following (1) What Wildman *et al* observed was that the amount of a normal protein present in the leaf declined during the time when TMV was synthesized they assumed a causal connection between these two events However their data show that the normal protein content of *uninfected* leaf also declined during the time of the experiments There is therefore in their own data no support for the conclusion that TMV is necessarily formed from normal protein (2) Isotope experiments by Meneghini and Delwiche and in my own laboratory show that the bulk of TMV nitrogen must be derived from a non protein source In our own experiments there is specific evidence that the nitrogen appearing in TMV does not pass through the soluble normal proteins the bulk of which is due to the protein which Wildman *et al* proposed as the source of TMV (3) Repeated tests show no antigenic relationships between TMV and the supposed normal protein precursor It is quite possible of course especially in conditions of nitrogen starvation that nitrogen originally contained in normal proteins may serve as a source of TMV nitrogen However this can only occur as a result of degradation and the normal protein nitrogen must then pass through the non protein nitrogen pool before becoming available for TMV synthesis A normal protein which so serves as a source of TMV nitrogen does not have a specific role in TMV synthesis

DR RIVERS There are substances known as specific soluble substances associated with viruses and for a number of years my associates worked on the specific soluble substances of vaccinia I have never been able to convince myself that this specific soluble substance was other than something made by a sick cell that was infected by vaccinia virus I don t believe anybody has brought evidence to prove that specific soluble substance is an essential part of vaccinia virus I know that many people believe that it is a part of the virus but the proof has never been brought Now I d like to ask a simple question When a diphtheria bacillus is infected with a prophage it makes

diphtheria toxin I ask Dr Commoner whether or not diphtheria toxin is in any way related to the precursor of prophage

MR BAWDEN I would like to comment on Dr Rivers question and say that in my opinion the various specific antigens from plants infected with tobacco mosaic virus are serologically equivalent to the soluble antigens that have been found associated with some animal virus diseases All of them can be considered as products of a deranged protein metabolism of the host and whereas the structure of some fits a pattern that makes them infective particles the structure of others does not

Dr Horsfall asked whether the various antigens are identical or only related I cannot answer for Dr Commoner s fractions but when Pirie and I were fractionating the anomalous nucleoprotein from tobacco mosaic plants we found that we could get a range of particles with sedimentation constants ranging from about 20 to over 200 The large particles were greatly elongated and gave serological reactions of typical flagellar type whereas the small ones were almost spherical and gave typical somatic reactions Their antigenic content however seemed to be identical for we prepared antisera separately against preparations of the largest and the smallest particles and each antiserum could be absorbed completely of antibodies by each type of preparation When large particles are disrupted by ultrasonics too the antigenicity is not altered Schramm considers that every antigen characteristic of each strain of tobacco mosaic virus is carried with each *fraction of the virus with a molecular weight of about 30 000* Antigenically then there seems to be no difference at all between the large and small specific particles found in extracts from plants with tobacco mosaic but there is a world of difference in their ability to infect healthy plants It is this difference the distinction between a protein that is and is not infective that separates plant viruses from other nucleoproteins and is one we need to find for in it may lie the origin of virus diseases

Part II

Ecology and Pathogenesis of Virus and Rickettsial Infections

Moderator

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13

Ecology and Virus Reservoirs

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Introduction

Because of the large shortage of knowledge concerning the natural history of virus diseases my discussion of the topic that has been assigned me Ecology and Virus Reservoirs will have to be partly speculative I shall begin with the thesis that we do not understand completely the ecology of any single virus disease of either animals or man though we do have reasonably accurate knowledge of the epidemiology of some of them We understand for instance the epidemiology of yellow fever dengue and equine encephalomyelitis encompassed in the host to-host transmissions of the causative viruses by mosquitoes We are lacking however in full knowledge of the eventual sources from which these viruses come to set up and establish that segment of the cycle that we do understand In the cases of the contagious virus diseases of man we have a good understanding for instance of the transmission of measles mumps and chicken pox from case to case once an epidemic gets under way but are completely lacking in accurate information as to the exact sources from which the virus infecting the first case in an outbreak may have come Even in the case of a virus disease such as rabies whose epidemiology we understand so well we are still woefully lacking in accurate information concerning its ecology and the mechanism by which a reservoir of infection is maintained in nature

For reasons that are readily apparent to anyone who has tried to do epidemiological or clinical research with human material animals and their diseases present a much more favorable subject for study than do man and his ailments It is probably because of this that we have more extensive information of an ecological character concerning several animal virus diseases than we do concerning human virus infections While as mentioned in an earlier paragraph I do not consider that we understand completely

the ecology of any single virus disease of either animals or man there are several animal virus diseases in which I believe we have a sufficient understanding of the ecological factors involved to serve for profitable discussion I should like therefore to consider three virus diseases of animals which are of interest from the standpoint of the role played by reservoir hosts in their ecology and which seem to me to illustrate points that may profitably be applied in working out the ecology of other virus infections less approachable from an experimental standpoint Each of these three diseases will be outlined primarily from the standpoint of the progressive steps which led to the elucidation of its ecological features to the stage at which these are now understood or assumed

Consideration of Ecological Patterns in Certain Virus Diseases

Bovine Pseudorabies This may be defined as an acute highly fatal infectious disease of cattle caused by the pseudorabies virus The disease is known popularly throughout the Middle West where it is most prevalent as mad itch from its cardinal clinical feature an extreme pruritis in which the animals mutilate an area of skin somewhere on their bodies by persistently licking and biting at the affected area [1] Death always ensues usually within 36 to 48 hours of the time the animal is first noticed to be affected As a rule only a small portion of a herd is involved and it is not uncommon to have single cases observed in rather large groups of animals Bovine pseudorabies is not a contagious disease and normal cattle can be pastured or stabled in intimate contact with sick cows without hazard of infection *Each case of bovine pseudorabies constitutes in reality a blind alley infec*tion and it is obvious that unless some reservoir host capable of perpetuating the virus existed pseudorabies would die out and be forever lost in the first cow it killed

Consideration of several outbreaks of the disease on Iowa farms made it quite apparent that the cattle were being infected by exposure to some host normally present in the barnyards Since in these various outbreaks only cattle among the several types of animals resident in the barnyards died it was apparent that the infecting host whatever it might be must be serving as a reservoir host one not subject to fatal or even clinically recognizable infection by the pseudorabies virus Cats dogs horses sheep and rodents could be eliminated from consideration on this basis because they could all be shown to develop fatal pseudorabies when infected with the virus It was studies of the pathogenicity of the virus for swine that finally furnished the clue to the reservoir host for bovine pseudorabies It was found that pseudorabies in pigs was quite different from that in any of the other animal species studied Instead of regularly killing as it did in cattle and all of the other potentially suspect hosts pseudorabies virus caused an extremely mild and almost silent infection in swine Aside from a transient temperature elevation swine showed few clinical manifestations of illness Furthermore

pseudorabies in swine proved to be highly contagious in contrast to its non contagiousness in cattle and other experimental animals Its mild but highly contagious character thus fitted it potentially at least as an ideal reservoir infection It was found that transmission from swine to swine was by way of the nasal passages and in some instances virus could be detected in or on the noses of infected pigs for as long as 10 days Pseudorabies could be transmitted from swine to experimental animals by mere nose to skin con tact and it is believed that this is the mechanism of transfer from swine to cattle under natural conditions

Two field observations supported and strengthened this hypothesis One of these was the observation that all cases of bovine pseudorabies seen per sonally or of which I had a record occurred on farms where swine and cattle were kept together in the same enclosures The other was the obser vation that in two outbreaks where the matter was studied it was found that pseudorabies virus neutralizing antibodies were present in the sera of swine associated with the infected cattle This indicated that the swine had under gone a previous infection with pseudorabies virus and could therefore have been responsible for the spread of the virus to the cattle with which they associated A point of some interest was that on all farms where pseudo-rabies occurred among the cattle no illness was noted among the swine by the owners the porcine pseudorabies infection had apparently been com pletely silent

With the incrimination of swine as the reservoir host from which cattle can acquire pseudorabies at least a partial understanding of the ecology of bovine pseudorabies was at hand That the whole of its ecology has not been clarified is indicated by the fact that we do not as yet know the mech anisms by which pseudorabies virus is maintained in its porcine reservoir host While the virus produces a contagious disease in swine and persists in the noses of infected animals for a number of days it seems questionable that case to case transfer can be held accountable for maintaining the in fection permanently in a swine drove It seems most probable that some other reservoir host capable of periodically setting up porcine outbreaks exists on infected farms The nature of this hypothetical reservoir is not known There is a possibility that infected rats migrating to another barn yard to die can when eaten by swine on the new farm establish a new porcine reservoir [3] However since infected rats always die within a period of 2 or 3 days of infection they cannot be visualized in any epidemiologic role other than that of a potential mechanism for spreading pseudorabies from swine drove to swine drove on neighboring farms and certainly could not be held suspect of maintaining a permanent swine reservoir of infection

The factors which have led to the partial elucidation of the ecological features of bovine pseudorabies may be summarized as follows

1 The opportunity to study outbreaks of the disease *repeatedly in rela tively* closed populations of cattle

2 The finding of a host swine in which pseudorabies virus causes a mild subclinical ailment

3 The finding that this potential reservoir host sheds virus in a manner capable of infecting cattle

4 The observation that all outbreaks of bovine pseudorabies occur on farms where swine and cattle are kept together in the same enclosures

5 The observation that on farms where bovine pseudorabies occurs the associated swine furnish serological evidence of having undergone infection with pseudorabies virus

Salmon Poisoning This is an acute highly fatal disease of dogs and other *Canidae* limited geographically in distribution to that portion of north western California western Oregon and southwestern Washington lying west of the Cascade Mountains The disease has been recognized ever since the Pacific Northwest was first settled by white men and their dogs but a series of varying views concerning the cause of the condition have marked the working out of its ecology Practical dog owners living along the streams and rivers of the area in which the disease prevails had long contended that it was a definite clinical entity which resulted when their dogs ate spawned out salmon and it was for this reason that the condition came to be known as salmon poisoning [4] It was not until 1927 when Donham[5] reported finding an intestinal fluke now known as *Troglotrema salmincola* in dogs dying of the disease that the first real progress was made towards understanding the disease and determining its cause Donham found the encysted metacercariae of this fluke in both salmon and trout produced the disease by feeding these metacercariae to dogs and recovered the ma tured parasites from his experimentally fed animals He believed at the time that his experiments indicated this fluke to be the actual cause of salmon poisoning Later it was shown that the snail host of the fluke was *Goni obasis plicifera* This discovery explained the geographical distribution of the disease as the snail in question has been collected only in those sections where salmon poisoning occurs [6]

For about 5 years Donham s fluke seemed satisfactorily to fill the role of the etiological agent in salmon poisoning However certain features of the disease were incompatible with the fluke theory of causation and it was these that led to further work and a clearer understanding of the actual role played by the fluke It was recognized by people working with the disease that it had many characteristics of an acute infection The definite incubation period the sudden onset the severe systemic reaction rapid course and the definite immunity in those few dogs which recovered were all suggestive of an infectious disease The crucial experiments were published by Simms and his co workers in 1932 and in these it was demon strated that an infectious agent separable from the fluke was capable of reproducing the clinical picture of salmon poisoning It was found that

blood taken from dogs acutely ill following the ingestion of fluke infested fish contained an agent that was transmissible serially to other dogs Such blood given either subcutaneously or intraperitoneally to dogs caused a disease that was identical to that resulting from the ingestion of parasitized fish The infectious agent which was not cultivable on bacteriological me dia could be passed successfully from dog to dog by blood transfer It was apparent from this work that the true cause of salmon poisoning was an infectious agent carried by the worm but separable from it The causative agent has recently been determined and from its description appears to have the properties of a large virus like entity [8]

With the incrimination of the fluke *Troglotrema salmincola* as a reser voir host for the causative agent a partial understanding of the ecology of salmon poisoning was at hand The cycle through which the virus had to pass in getting from dog to dog was a complicated one and one in which as will be pointed out later gaps in our knowledge still exist

To recapitulate and summarize the ecology of salmon poisoning the survival of the virus and its transmission from dog to dog is apparently dependent upon infected *miracidia* shed by flukes within the gut of a sick dog getting to a particular species of snail *Goniobasis plicifera* After de velopment within this snail cercariae are shed by the snail and these infest either a salmon or a trout Within the fish the cercariae penetrate to an appropriate organ usually the kidney where they encyst and become meta cercariae The metacercariae after ingestion by a dog in the process of devouring an infested fish develop to adult flukes within the gut of the dog and in the process infect the dog with the salmon poisoning virus that has been carried throughout the whole of the worm s cycle from dog through snail and fish and back to dog

As mentioned above there are still gaps in our knowledge of the cycle One of these concerns the physiological state of the virus within the redial and cercarial developmental stages of the worm in its snail intermediate host In these two worm stages virus has not been demonstrated directly by dog inoculation[7] and it seems likely that it exists in a masked or non infective form In the metacercarial stage in fish on the other hand and of course in the adult fluke in its dog definitive host virus is directly demon strable by infectivity tests Another gap in our knowledge of the ecology of salmon poisoning concerns the definitive mammalian host other than ca nines in which the virus infected fluke can live without killing its host Such a host must exist to assure perpetuation not only of the fluke but of the salmon poisoning virus that it carries as well Without such a host to main tain the cycle the high fatality of the virus for canines could under natural conditions be expected to break it at the definitive host stage with conse quent eventual disappearance of the disease through a process that might aptly be termed ecologic suicide The raccoon may be the alternative

definitive host for the virus infected fluke[9] and perhaps this host will eventually be established as an important ecological factor in the natural history of salmon poisoning

The factors which have led to the elucidation of the ecological features of salmon poisoning to the point that these are understood today may be summarized as follows

1 Recognition that the disease was limited to a circumscribed geographical area and was in some way related to the ingestion of fish

2 The finding of a parasitic worm in dogs common to all cases of the disease

3 The identification of the metacercarial stage of this worm in fish

4 The observation that the snail intermediate host of the worm had a geographical distribution coinciding closely with that of the disease itself

5 The recognition that salmon poisoning presented a clinical picture differing from that expected to result from a worm infestation *per se*

6 The establishment of the fact that the disease could be transmitted serially in dogs by an agent separable from the worm

7 The identification of the causative agent

Swine Influenza *This is an acute highly contagious respiratory disease* of pigs caused by *Hemophilus influenzae suis* and the swine influenza virus acting in concert The disease made its first appearance so far as anyone knows in 1918 at the time of the great human influenza pandemic [10] Any claim that a disease is entirely new usually arouses skepticism However the establishment of an infectious agent in a new host has often been carried out experimentally and might conceivably under favorable conditions occur in nature There is considerable indirect evidence that such a thing happened in the case of swine influenza and that the disease arose in pigs as a result of their infection by the virus which raged through the human population during the great 1918 influenza pandemic Laidlaw[11] and I[1] outlined some time ago evidence supporting that contention Recently Davenport Hennessy and Francis[12] have presented serological evidence adding strength to the hypothesis

This new disease which appeared in swine back in 1918 was not a sporadic and localized outbreak actually millions of hogs became ill and thousands died during the first few months that it prevailed The first epizootic persisted in various localities until January 1919 and reappeared in the autumn and winter of that year almost as extensive and severe as in 1918 It has recurred each year since then but varies annually in its severity and extent

Characteristically swine influenza epizootics begin explosively late in October or early in November [10 14] The build up in cases is extremely rapid and one gains the impression that the disease either spreads like wildfire or has arisen at many different foci simultaneously The disease if it is actually disseminated from farm to farm gets spread about in miraculously rapid

fashion in fact so rapidly that the theory of spread from farm to farm by actual contact is an almost unbelievable hypothesis After the initial wide spread outbreak fresh swine droves become infected in smaller and smaller numbers until by late December as a rule the epizootic appears to have run its course and swine influenza disappears as a farm infection until the following October or November The bacterial component of the etiological complex *H influen ae suis* can persist indefinitely in the upper respiratory tracts of some recovered swine However similar persistence of the virus cannot be demonstrated

The whereabouts of swine influenza virus during interepizootic periods and the origin of that infecting the first cases in a succeeding epizootic remained a mystery for many years While clues indicating that it must persist right on the affected farms from year to year were numerous they were not explicit enough to enable one to determine just which of the possibilities might be most profitable for exhaustive study The observation which finally led to an understanding of the epizootiology of swine influenza was made in two stages First it was found that swine under natural conditions could be carriers of latent swine influenza virus which by certain manipulations could be provoked to infectivity [1] Secondly it was found that swine acquired these latent swine influenza virus infections through an intermediate host the swine lungworm [16]

The intermediate host system whereby swine influenza virus from one epizootic is preserved to infect the first cases of a succeeding epizootic is not a simple one The swine lungworm is the actual reservoir and intermediate host of the virus However the lungworm has an intermediate host of its own the common earthworm and must spend three of its developmental stages in its earthworm intermediate host before it can parasitize swine [17,18] Swine acquire their lungworms by eating earthworms infested with third stage lungworm larvae If these lungworm larvae were hatched from eggs laid by an adult lungworm while she was infesting the lung of a pig with influenza they will be carriers of masked influenza virus The pig acquiring the larvae will then become infected with masked virus when the larvae migrate to its respiratory tract The term masked is applied to virus in its lungworm intermediate host to indicate that it is present there in an occult and not directly detectable form Swine infested with lungworm carriers of this masked swine influenza virus remain normal to all appearances and there is no way of detecting directly that they are actually carrying swine influenza virus However such swine are in a precarious situation so far as their eventual well being is concerned because all that is required to bring them down with a severe or perhaps even fatal attack of influenza is the application of some stimulus of itself relatively harmless Several such provocative stimuli have been used but the one that has proved most regularly effective consists in the administration of multiple intramuscular injections of the bacterium *H influen ae suis* [19] Under natural conditions

on the farm the provocative stimulus is probably meteorological in character and is in some way associated with the onset of wet cold inclement weather I succeeded last year in provoking influenza infections by placing prepared swine in pens on the roof of the Rockefeller Institute on cold snowy nights Similarly prepared swine kept indoors remained normal

The intermediate host mechanism that I have briefly outlined adequately accounts for the perpetuation of swine influenza It has been found that virus can persist for at least as long as 32 months in third stage lungworm larvae in their earthworm intermediate hosts and for at least an additional three months in association with adult lungworms in the swine respiratory tract [19] This constitutes a total elapsed time between the case of swine influenza originally supplying the virus and the hog eventually becoming infected with it of almost 3 years and is roughly three times the amount which must be accounted for to explain the survival of the virus from one epizootic to the next

There is evidence from some field work on the subject that the incidence of swine infected with masked virus may be quite high [8] This would suggest that the apparent paradox of swine influenza spreading throughout a drove and from farm to farm faster than we realize it can on the basis of any known incubation period may not be paradoxical at all Instead of the virus going like wildfire the field evidence indicates that it is probably widely seeded before the outbreak and merely provoked almost simultaneously The great rapidity of spread is therefore more apparent than real and represents a delusion resulting from the provocation of widely disseminated masked virus by a stimulus common to large geographical areas I do not mean to imply by this that swine influenza does not ordinarily behave like a typical contagious disease in spreading from sick to well animals I am certain that it does and in the laboratory under experimental conditions it can be shown to be regularly and highly contagious The point that I should like to emphasize however is that in swine influenza the number of first cases responsible for initiating an epizootic may be larger than is the rule in most other contagious diseases and that this may account for its high morbidity rate and for the thoroughness with which it involves an entire swine population

There are still a number of gaps in our knowledge of the ecology of swine influenza For instance the mechanism by which influenza virus is maintained in man the host that appears to have been the primary reservoir from which swine acquired their infection in the first instance is unknown Furthermore in this connection it is a little puzzling that the 1918 human influenza virus apparently established itself with such ease as a porcine infectious entity while subsequent invasions of swine by human influenza viruses have apparently not resulted in the establishment of these more recent strains as permanent porcine pathogens For instance the infection of swine on farms in New Jersey in 1936 with the type A influenza virus

then prevalent in man caused only a subclinical infection in the swine and did not permanently establish itself in the involved swine droves [21] It seems likely that although influenza virus may frequently leak from its human reservoir to swine something peculiar to the 1918 virus enabled this particular strain to become established in swine lungworms and to persist in this reservoir as a source of infection for pigs year after year While it may appear a little unusual to be concerned about the role played by man in the ecology of an animal disease actually until we understand the mechanism by which influenza virus is perpetuated in humans its apparent primary reservoir host so far as swine are concerned we cannot claim to understand the ecology of swine influenza in its entirety

Other gaps in our knowledge of the ecology of swine influenza concern the physiological state of the virus when masked in its lungworm reservoir host and the nature of the stimulus that provokes the masked virus to infectivity More exact knowledge of the phenomenon of masking would greatly clarify our understanding of that phase of the ecology of swine influenza involving the sojourn of the virus in its secondary reservoir host the swine lungworm It is lack of knowledge of the exact mechanism of this phase of the natural history of the disease that appears to present the gravest objection to the general acceptance of the role of the swine lungworm in the ecological setup of swine influenza Andrewes has voiced very aptly in his 1949 Dunham lecture on epidemic influenza the misgivings that many appear to have concerning the wholehearted acceptance of the mechanism He wrote as follows One thing worries me about Shope s experiment such a complex association between creatures of four species has the hallmark of an ecologic happy family the result of eons of evolution Yet neither the pig nor the earthworm concerned is native to the United States and swine influenza is asserted never to have been known in the Middle West before 1918 It would be odd if swine influenza which is not known to survive so long anywhere else (except in the virologist s dry ice container) should just happen to find the interior of a lungworm an ideal resting place

I am personally not convinced that the development of an ecologic happy family always requires eons for its evolution and certainly recent events strongly suggest that the myxoma virus completely foreign to Australia only a few years ago is finding things down there very much to its ecological liking despite the fact that it is in brand new territory [3] To judge from the manner in which it is performing and persisting in its new environment it has found intermediate hosts and a satisfactory reservoir host at present not well understood without apparent evolution of any of the various factors which must be involved in its ecology

I would agree with Andrewes that it is odd indeed that the influenza virus should with no known evolutionary developments suddenly establish itself comfortably and apparently permanently in an ecological arrangement in

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mind of using these as examples to indicate possible future profitable courses that might be followed in working out the ecologies of other virus diseases less readily accessible to experimentation Our knowledge of the epidemiology of most virus diseases of man and many of those of animals is today fairly complete but we lack the information which would enable us to understand their natural histories We know much about most of them while they are prevalent as disease entities in a population for instance but we are largely ignorant of where their causative agents disappear when the epidemics cease nor do we know where these agents come from to start new epidemics Knowledge of this phase of virus diseases is embodied in a more complete understanding of their ecologies and to me the term ecology applied in this way implies the existence of one or more reservoir hosts for each disease In the ecology of swine influenza for instance two reservoirs are involved one of these man might be termed the initiating or primary reservoir while the other the lungworm could be called the perpetuating or secondary reservoir host The primary reservoir host in this instance was responsible for starting the disease in swine in the first instance while the secondary reservoir host functioned in perpetuating it once it became established in swine In the case of bovine pseudorabies swine serve as the secondary reservoir host and as pointed out earlier we are still ignorant concerning the reservoir from which swine acquire their infections or the mechanism that perpetuates the disease in swine In salmon poisoning the fluke *Troglotrema salmincola* serves as the secondary reservoir host and it is possible that the raccoon may function as the primary reservoir

From analogy with those virus diseases about which we have the most complete ecological information it seems logical to think that in all that appear to be well established and native to a given geographical area reservoir hosts capable of perpetuating them will be found to play a major role in their ecology Those diseases that fail to become established fail for lack of a reservoir in which their causative viruses can persist from one outbreak to the next I can illustrate what I mean by citing three hypothetical examples

In the first of these the case of myxomatosis in rabbits presently prevailing in Australia only a secondary or perpetuating reservoir host appears to be involved The primary or initiating reservoir in this case was man with a hypodermic syringe and this can hardly be counted as a normal factor in the ecology of a disease

The second example that may be cited is that of hog cholera in the United States Here both a primary and a secondary reservoir host for the hog cholera virus must have been involved though neither are known even today Hog cholera started in this country in a very gradual and insidious manner [24] The first outbreak appears to have been in Ohio in 1833 In 1837 an outbreak occurred in South Carolina The following year the disease appeared in Georgia and in 1840 single counties in Alabama Florida Illi

volving swine earthworms and lungworms However, I should hope that Andrewes would agree with my view that actually this arrangement with regard to influenza is no more odd than is the current myxoma situation in Australia in which yet another virus has suddenly and without apparent evolutionary developments found itself in an ecological happy hunting ground

In closing my discussion of swine influenza I should like briefly to outline the factors which have led to a partial elucidation of the ecological features of this disease They are as follows

1 The apparent origin of the disease at a definite point in time and co incident with an outbreak of a similar disease in man thus indicating that the probable primary reservoir host from which swine acquired their in fection in the first instance was man

2 The recurrence of the disease each year since 1918 at many different foci simultaneously suggesting multiple points of origin of the annual epi zootics and the probable existence of a reservoir host other than man capable of perpetuating the infection

3 Circumstantial evidence strongly suggesting that on those farms having a history of annual recurrences of influenza in their swine droves this secondary reservoir of infection was some host normally resident on those farms

4 The regular seasonal incidence of the onset of swine influenza and the association of this onset with the beginning of inclement weather each autumn

5 The discovery that swine under natural conditions could be carriers of latent swine influenza virus which by certain manipulations could be provoked to infectivity

6 The working out and establishment of the swine lungworm develop mental cycle involving an earthworm intermediate host by the Hobmaiers and by Schwartz and Alicata

7 The discovery that the swine lungworm could under experimental con ditions serve as a reservoir and intermediate host for the swine influenza virus and that the virus in its lungworm host was in a masked or latent form

8 The observation that masked swine influenza virus that had been carried to the swine respiratory tract by migrating lungworms could by ap propriate manipulations be provoked to infectivity

9 The establishment of the fact by field experiments that the swine lungworm is frequently infected with masked swine influenza virus under natural conditions and serves as the reservoir from which swine can acquire their influenza virus infections each year

Discussion

I have outlined the factors leading to a partial understanding of the rather complex ecologies of three virus diseases of animals with the purpose in

important role for the primary reservoir and an increasingly important one for the secondary reservoir Like swine influenza which was to make its appearance many years later hog cholera was a disease of marked seasonal incidence However unlike swine influenza which was to prevail from mid October through December hog cholera ordinarily began in late July or early August and ran the major part of its epidemic course during the next 2 or 3 months While as mentioned earlier in this paragraph the existence of both the primary and secondary reservoir hosts in hog cholera are purely speculative no other hypothetical arrangement accounts for the historical facts of the disease as well as the assumption that two such reservoirs must exist The secondary reservoir in hog cholera indeed might from certain similarities existing between the epidemiological pictures of hog cholera and swine influenza be similar to that in swine influenza The primary reservoir host must be some animal with a geographical distribution peculiarly limited to those areas in which hog cholera made its early appearance during the first 15 or 20 years of its prevalence This host whatever it is has either a very low incidence of latent hog cholera virus infection or a very faulty and inadequate mechanism for transferring its latent virus to swine

The third example that may be cited is that of foot and mouth disease in the United States Here only a primary or initiating reservoir host seems to be involved and a secondary or perpetuating reservoir appears to be lacking Foot and mouth disease has been introduced into the United States six times since the turn of the century and each time it has been eradicated by procedures involving quarantines and the slaughter of all infected or exposed cattle and other cloven hoofed animals Each introduction was through the medium of infected animals or animal products vaccine lymph in one instance and raw garbage containing meat trimmings in two others These media containing foot and mouth disease virus constituted the initial source from which the cattle and swine in our country acquired their infections with foot and mouth disease and hence in the sense of this discussion constituted the primary reservoir of infection Once started the disease spread rapidly by contact Five of the six outbreaks were of relatively short duration and limited in geographical distribution One however that which started in 1914 prevailed for almost 2 years and spread into 22 states before it was eventually controlled and the infection eradicated Thus in six instances an opportunity was presented for the foot and mouth disease virus to become established in a secondary or perpetuating reservoir host in this country One of these opportunities spanned a period of almost 2 years and the geographical and climatological peculiarities embodied in 22 different states The fact that the disease did not become established seems a strong indication that no host capable of serving as a perpetuating reservoir for the virus exists in the United States

In citing the examples that I have I ve attempted to point out the importance of reservoir hosts in virus ecology by indicating the role that these

nois and Indiana recorded the occurrence of a swine disease thought to be cholera By 1845 the disease had made its appearance in three more geographical areas Thus in a period of 13 years only 10 widely separated out breaks of hog cholera had occurred It prevailed at that time in no other part of the world so far as can be learned The disease had all of the ear marks of an infectious process that was either repeatedly arising *de novo* or was being repeatedly transmitted to swine from some reservoir host present in the areas involved Since *de novo* origin of any infectious agent is in the present state of our knowledge difficult to visualize the best ex planation would seem to be that a reservoir host for the virus probably a wild animal indigenous to the areas involved was periodically and oc casionally transmitting hog cholera virus to swine That the virus was having a difficult time getting started in its new host is indicated by the slowness with which it became widely established in swine The indications are in the light of our later knowledge that each of those 10 outbreaks prior to 1845 were probably initial infections resulting from exposure to the virus of a primary reservoir host Nothing in this early history of the disease sug gests the establishment of the virus in a secondary reservoir host of the type capable of perpetuating it and carrying it over from year to year in a given locality However there was not long to wait for although hog cholera appeared to be a slow starter it made up for lost time once it got under way In the 10 years between 1845 and 1855 at least 90 more outbreaks were reported with extensive spread into new geographical areas These new outbreaks were probably partly the result of infection from the primary reservoir host but the fact that the incidence of the disease had increased over 9 fold as compared with its rate for the first 13 years of its prevalence strongly suggested that a secondary reservoir host had entered the picture to play its role The evidence indicated establishment of the hog cholera virus in a reservoir more facile in infecting swine than was the initial one The uniformly fatal character of hog cholera rather well eliminated from consideration the possibility that this secondary reservoir might be the pig itself Hog cholera after 1855 increased by leaps and bounds and rapidly became one of the most important livestock diseases with which this country had to contend By the 1880s the Bureau of Animal Industry estimates placed the losses from cholera at never less than $10 000 000 per year and sometimes reaching $25 000 000 annually By this time cholera was of annual occurrence and reappeared year after year in the same localities

It was quite apparent that by now the hog cholera virus was widely seeded throughout the swine raising states and had become firmly estab lished in a reservoir host capable of carrying it over from one year to the next and setting up new outbreaks each year on a widespread basis No doubt the primary reservoir host still accounted for the occurrence of some outbreaks but the marked increase in incidence of the disease over what it had been in its earlier period of prevalence indicated a diminishingly

yield leading information Certainly in studies on pseudorabies the observation that swine infected with the virus suffered only a mild almost asymptomatic illness that this mild disease in swine was contagious from pig to pig and that the virus was abundantly present in and on the pigs noses strongly suggested swine as a rather ideally equipped reservoir host from which cattle could acquire the disease

Another approach that may be of value in obtaining leading facts concerning the ecology of virus diseases is the search for unknown viruses in potential reservoir hosts This approach would be of no use in diseases such as salmon poisoning or swine influenza in which the viruses may be masked and hence not directly demonstrable by infectivity tests in their reservoir hosts However the investigation of wild mosquitoes by Roca Garcia Smithburn and his co-workers and others [5] have yielded mouse pathogenic viruses in numbers which someday may be fitted into the ecological setups in which they rightfully belong as causative agents for clinically manifest illnesses in as yet unsuspected and unidentified natural hosts Other biological forms such as leeches various biting insects aside from mosquitoes and the cercarial stages of trematodes all capable of surgically inflicting portals of entry through which viruses that they might adventitiously be carrying could gain access to a susceptible host have not been seriously investigated When they are examined it may well be that they will yield as rich a harvest of new and potentially important viruses (and some may even be recognizable) as have the tropical mosquitoes

Now just who will do all of this searching for virus reservoirs and the other field work that I have touched on in my discussion? It seems to me that this area of investigation should be a paradise for the naturalist and for those who want to study and follow the leads that nature supplies Undoubtedly much of the knowledge that might permit an informed and thoughtful individual to forecast profitable fields for investigation is already at hand However much of this knowledge is possessed by people with little or no interest in infectious diseases *per se* or is buried in literature with which the virologist is completely unfamiliar Work in virus ecology can probably be done more logically and effectively by having the naturalist turn virologist than by having the virologist attempt to acquire the extensive knowledge and experience possessed by the naturalist The field of virus ecology it seems to me is one of the few remaining in virology that can still be pursued profitably without recourse to the expensive bits of apparatus and the complicated laboratory techniques which are becoming so much a part of modern virology

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may play in determining the permanency of virus diseases in given geographical areas If both a primary and a potential secondary reservoir host exist in an area then one may expect a disease to become permanently established once a host capable of exhibiting clinical signs of illness is introduced and it seems possible that hog cholera represents such a situation If only a secondary perpetuating reservoir exists then the establishment of a disease on a permanent basis will be dependent upon the introduction of the pathogenic virus *from without* Swine influenza in this country and rabbit myxomatosis in Australia would seem to furnish examples in which this situation prevailed If no secondary perpetuating reservoir exists in an area containing a population susceptible to clinical infection by a virus then introduction of that virus from without will result in a sharp but probably limited outbreak of disease that will run its course until the susceptible population is killed or immunized and will then disappear until reintroduced from without Foot and mouth disease as it occurs in the United States would seem to furnish an example of this situation

What factors may lead to the determination of potential virus reservoirs and hence contribute to an understanding of the ecology of virus diseases? Several factors were very helpful in furnishing leading clues in the cases of the diseases I ve discussed in this paper If an outbreak or epidemic can be oriented as to place of origin an ecological clue may be furnished by such an observation For instance the fact that pseudorabies in cattle appeared as sporadic outbreaks on individual and frequently widely separated farms furnished an indication that the source of the virus must be some reservoir present on the affected farm itself Similarly the onset of swine influenza each autumn simultaneously in many different swine droves indicated strongly that the virus reservoir lay within the drove itself or in close association with it The limitation of salmon poisoning to an area populated by the snail *Goniobasis plicifera* and the discovery that this snail was an intermediate host for *Troglotrema salmincola* rather clearly could have furnished a clue as to the reservoir host for salmon poisoning though in fact the fluke had already been incriminated before its intermediate host system had been worked out

Orientation of a disease as to the time of its origin can under certain special circumstances be helpful in determining its ecology This was true in the case of swine influenza which made its first appearance in 1918 coincident with a human influenza outbreak

The finding of specific virus neutralizing antibodies in the sera of potential reservoir hosts can be of value in incriminating them ecologically The presence of pseudorabies virus neutralizing antibodies in the sera of swine pointed a finger of suspicion at this animal as a likely reservoir from which cattle might be infected

Testing various potential animal hosts for susceptibility to the virus whose ecology is being investigated is another approach that conceivably might

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infested rats rose rapidly from a few percent to 70 per cent Two or three months later there was a second even higher peak to some 80 per cent But whereas most infested rats at the time of the first peak had 10–50 chiggers per rat at the time of the second peak there was a sudden shift in distribution so that most rats had 200 or more chiggers There may have been two other smaller peaks the last a minor one in the dry season but data are insufficient to be certain of these All the evidence suggests that at the time of the first peak a large number of fed larvae were returned over a short time to the soil for further development The second peak represented the completion of the life cycle and perhaps the later peaks were adumbrations of the earlier ones (Audy 1947) Allowing for a feed ing time of 3–4 days we may hazard a rough calculation and say that dur ing the six dry months December to May the total number of mites which passed through their larval stage was about 200 for each rat Over the six wet months June to November the corresponding turnover was over 2 000 per rat During the peak months of September and October the turnover was of the order of 650 chiggers per rat per month while during one of the dry months it went as low as 20

Secondly there are periodic fluctuations in the populations of animal hosts of which rats are the most prominent We have no good observations of these from our own work but there are many examples from elsewhere One example in Asia is that of the plague of rats which follows the mass flowering and fruiting of gregarious bamboos in parts of India and Burma —a huge tract may fruit and die down to be followed by a plague of rats usually leading to famine and the abandonment of villages

Thirdly there are the small local fluctuations which occur as discrete space time events whenever a patch of land is allowed to go to waste and become overrun by weeds The weeds themselves of course represent large population fluctuations of certain plants and they are accompanied by equally exuberant animal weeds such as rats The growth of such local concentrations takes time and has not actually been followed through by our own observations All stages have been observed however by sampling many such areas and the neighbouring forest

Fourthly in some local concentrations of vector mites a high degree of rickettsial infection can be detected but in other concentrations there may appear to be no recoverable infection (I am here drawing upon the massed experiences of many workers) The rickettsiae evidently undergo consider able population fluctuations subject to their own particular environmental influences

Finally I would draw your attention to a gigantic population upheaval which has generally escaped notice because it has a very different tempo It is that of *Rattus rattus* and its immediate relatives in southeast Asia This rat occurs in largest numbers outside the native forest in artificial terrain which follows deforestation and settlement The centre of development of

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The Effect of Host and Vector Densities on the Epidemiology of Scrub Typhus

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In early 1945 an Army laboratory at Imphal on the Indo Burma border started investigating what seemed to be a very significant association that of hyperendemic scrub typhus with deforested land overrun by only one or two species of wild rat Endemic scrub typhus appeared to be largely a man made disease occurring in artificial terrain and the close parallel with urban plague and epidemic yellow fever led to the suspicion that the disease had escaped from a reservoir in the mature forest which was the original natural vegetation The name jungle tsutsugamushi was coined to describe the uninvestigated wild infections which were presumably being obscured by the tremendous secondary development of infection in scrub Such a jungle infection has been demonstrated by Traub Frick and Diercks in Malaya (1952) but as I hope to make clear later there may be less obviously virulent rickettsiae in the tsutsugamushi group native to the forest and these have hardly been investigated

The epidemiologist is deeply concerned with population fluctuations at first of pathogens and then of vectors and animal hosts or reservoirs I shall here almost confine myself to the causes and effects of population fluctuations of vectors and hosts in relation to pathogens especially of course rickettsiae I should like to summarise several observations which are matters of fact and will give us a basis for discussion

Firstly the vector mite *Trombicula deliensis* was found in Imphal to be very sensitive to dryness The larvae had a sharply seasonal incidence coinciding with the rains After the rains started in May the proportion of

* Including the Scrub Typhus Research Unit supported by Colonial Development & Welfare Funds

Table 2

THE PATTERN OF INFESTATION OF THE COMMONER RODENTS IN SELANGOR MALAYA BY THE CHIEF GROUPS OF TROMBICULID MITES TRIVIAL INFESTATIONS ARE REPRESENTED BY DOTS (CERTAIN SPECIES E G THE SPINY RATS AND *R exulans* SHOWN HERE AND *R cremoriventer* O ITTED FRO I THE TABLE ARE VERY LIGHTLY INFESTED BY CHIGGERS IN ALL OUR MALAYAN COLLECTIONS LARGER SUBSPECIES OF ■ *exulans* IN THE PACIFIC AREA ARE OFTEN VERY HEAVILY INFESTED BY THE VECTORS)

SPECIES OF HOSTS AND NUMBERS EXAMINED	MEAN NUMBER OF TROMBICULIDS PER INDIVIDUAL					
	Total all species	*Vec tors T deli ensis aka mushi*	*Eusch oude mansi lacu nosa*	*Eusch indica a idyi*	*Gahr liepia s lat*	*Other spe cies*
IN FOREST RATS AND SQUIRRELS						
Tree squirrels (*Callosciurus cani ceps nigrovittati s notatus te nuis*) 664	24			23		
Tree rat (*R canus*) 11	38	0	11	5		22
Ground squirrels (*Rh nosciurus* & *Lariscus*) 29	31	10	10		4	7
Ground rats						
Giant rats (*R bowe s muller sabanus*) 678	26	7	11		4	4
Spiny rats (*R pellax si rifer whitehead*) 63	6				5	
OUTSIDE FOREST , COMMENSAL RATS						
R r j lorensis						
(mature oilpalms) 1,288	49	11		37		1
(open woody scrub) ?41	?	15		4		3
R • argenti enter (open scrub) 387	81	78		1		2
R r subspp (3 islands) 50	40	140	0	0	100	0
R r diard (town) 2 430	19	0	•	19	•	
R norveg cus (seaport) 8_4	10	0	0	10	•	0
R exul ns concolor (open scrub) 650	?	1	0	1		

The Emergence of Scrub Typhus from Jungle Tsutsugamushi

In the case of both the chief host or animal reservoir and the mite vector or primary arthropod reservoir of a rickettsia we therefore have two fea tures viz great abundance and local population fluctuations These two features are known to be of great evolutionary importance An abundant species draws upon a relatively much richer store of inheritable variation which gives it a capacity to undergo adaptive changes Also a large popula tion fluctuation especially if starting from a very low level can allow un usual changes in genetic constitution For example chance mutations which would be swamped in more stable populations may in these conditions of

the genus is in southeast Asia where over 500 named forms of *Rattus* have been recorded The species *R rattus* has developed enormously and has been dispersed from this centre all over the world as the black rat or ship-rat Some 46 forms of this species have been named in Malaysia This great development appears to have been secondary to deforestation and settlement The prime importance of *Rattus rattus* in connexion with scrub typhus has been discussed elsewhere (Harrison & Audy 1951)

It is important to note the relatively greater abundance of individuals of those species of rat which occur in the deforested areas This is suggested by the data in Table 1 while Table 2 shows the corresponding great increase in the numbers of a few trombiculids including the vectors parasitising these same animals

Table 1

NUMBERS OF SPECIMENS OF SMALL MAMMALS MARKED AND TRAPPED ON THE GROUND IN (A) A 10 HECTARE (25 ACRE) AREA OF DISTURBED FOREST AND (B) A 10 HECTARE AREA OF GRASSLAND WITH SCRUB (UNPUBLISHED DATA J L HARRISON)

RODENTS—RATS	*Forest*	*Grassy scrub*
Rattus exulans	4	152
R rattus jalorensis	0	28*
R r argentiventer	1	24*
R whiteheadi	35	20
R pellax	41	0
R surifer	33	0
R mulleri	23	0
R sabanus	8	0
RODENTS—SQUIRRELS		
Rhinosciurus laticaudatus	1	0
Callosciurus tenuis	4	0
C notatus	4	0
C nigrovittatus	1	0
INSECTIVORES		
Echinosorex gymnurus	1	0
Tupaiaglis	1	0
Total individuals	159	224
Total species	14	4
Total number of species of mammals known to occur in the area	66	16

* *R r jalorensis* occurs in lightly wooded scrub while *R r argentiventer* has a very strong preference for open grassland In this particular patchwork investigated neither species appears to advantage

Four examples have been given of population density changes taking place in the hosts vectors and rickettsiae of endemic scrub typhus The changes have been on different time scales from the sudden fluctuations associated with seasons to the slower and greater changes ascribed to deforestation Tabulated data have been added to suggest fundamental changes in the composition of forest following clearing

ing hypotheses for guidance and in the case of population instability we require one which is concerned with the questions when and where among living things we do get concentration instead of dispersal and imbalance instead of numerical stability?

The equatorial rain forest is the richest association of living things to be found on land It occurs in stable climatic conditions of heavy perennial rainfall and unchanging high temperature It is characterised by a tremendous richness in species—there may be 100 species of trees and 30 species of mammals in a single acre of rain forest With this richness goes dispersal individuals of any one species tend to be scattered and interspersed with other species The effect of this on the balance of populations is closely similar to that of a buffer solution any fortuitous fluctuations are readily damped down by the other components of the balanced system We may therefore look upon the rain forest as a formation with great internal stability

This stable formation gets simplified in nature either by changes in the uniform climate which nurses it or by its physical destruction As one goes to higher latitudes at first one season and later all seasons become increasingly rigorous Increasing altitude has a similar effect Destruction of the forest leaves bare areas which become invaded by a few quick growing plants and a few animals and it may be many years before forest regenerates With repeated or extensive destruction the original conditions may never return and the forest may even be replaced by semipermanent grassland or savanah Table 3 shows the effects of deforestation in the neighbourhood of our laboratory in Kuala Lumpur but the table does not include the relatively greater abundance of the few species which exploit the new conditions (see Tables 1 and 2) Whatever the cause the effect of the simplication of the rain forest formation is to reduce the number of species greatly to increase the absolute numbers of many of the existing species and to allow concentration instead of dispersal so that individuals of various

Table 3

DISTRIBUTION OF SPECIES OF HOSTS AND MITES COLLECTED IN SELANGOR STATE MALAYA (CF TABLE 1 AUDY & HARRISON 1951)

Source	Rodents & Insectivores			Trombiculid Mites	
	Number trapped (to 1951)	*Number of Species*	*Number of Genera*	*Number of Species*	*Number of Genera*
Forest	5 574	49	30	[illegible]	6
Deforested areas	3 459	15	8	16	5
Town (houses in Kuala Lumpur)	6 813	5	3	[illegible]*	2

* Includes presumably introduced species

rapid change spread through a high proportion of the population leaving its genetic constitution modified

The operation of these two factors may presumably be seen in the fact that taxonomists are still battling with the great variation shown in both *Rattus rattus* (46 named forms in Malaysia) and the forms (over 6) of the vectors of scrub typhus

Man has deforested enormous tracts in Asia and we have noted that *Rattus rattus* has been able to exploit the new conditions of scrub and cultivated land (a patchwork which has been discussed as *parang* vegetation by Audy 1949) These new conditions have also been suitable for the development of field dwelling trombiculid mites of the *Trombicula akamushi* species-complex and the rat and the mite have together colonised the deforested areas This *R rattus* + *T akamushi* (*sensu lato*) association represents large compact rat populations and a great turnover of mite larvae It would have offered great opportunities for the forest enzootic rickettsia *Rickettsia tsutsugamushi* or a precursor to become adapted and to flourish In these conditions the infection emerges from the forest to develop freely in waste land and is then brought sharply to our attention as endemic scrub typhus

Cradled in the complex network of the *R rattus* + *T akamushi* association with innumerable collateral contacts with other animals and parasites one would expect the rickettsia to show much variation Some strains of *Rickettsia tsutsugamushi* are in fact known to differ from each other more widely than does *R prowazeki* from *R mooseri* Doubtless even greater differences will be found when the reservoirs of jungle tsutsugamushi are more thoroughly explored The point I wish to make here however is that it would appear that we have a rickettsiosis or a complex of rickettsioses native to the forest which has been offered an opportunity for special development and adaptation by a very large scale biological event namely the great development of the *R rattus* + *T akamushi* association in south east Asia This event has virtually evoked endemic scrub typhus There is no reason to suppose that the primitive jungle rickettsiosis has not the great antiquity of a related almost world wide rickettsiosis of ticks which has evolved in various local host parasite associations into the geographical forms of tick typhus and spotted fever We should therefore be prepared to accept the possibility that jungle tsutsugamushi may also represent an almost world wide rickettsiosis which may here and there have evolved locally along avenues offered to it The organism from *Trombicula microti* and voles in Canada reported by Baker (1946) may be an example of this

Discussion

Epidemiologists are now delving deeply into the enormously complex web of animal hosts or reservoirs and their many parasites In this we need all the ideas and methods of the animal ecologists We also need work

This it is suggested has the same effect as that of a buffer solution in damping down fortuitous population increases This complex is interfered with in nature by changes in the uniform climate which nurses the rain forest (e g in higher latitudes or altitudes) or by deforestation Whatever the cause the effects are the same fewer species are found but in relatively greater numbers and individuals of various species become concentrated into pure stands colonies herds and so on The increased abundance and local concentrations of relatively few species is inevitably accompanied by imbalance

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species form pure stands (e g weeds bamboos) colonies packs herds and so on With this increased abundance and irregular concentration of a relatively few species goes imbalance and cyclic population fluctuations as well as erratic local density increases and "crashes" These conditions encourage every kind of pestilential outbreak from mouse plagues to epizootics and blights The epidemiologist cannot get a full perspective without seeing man against this background—gregarious man who gathers himself into dense communities and who is constantly interfering with natural balances which can remain balanced only if their complexity is preserved

Summary

The epidemiologist is deeply concerned with the causes and effects of population fluctuations of pathogenic organisms and their vectors and of animal hosts or reservoirs Examples are given of such fluctuations in the populations of vector mites rats and rickettsiae and evidence is given of the abundance of field rats and vector mites The development of the genus *Rattus* and especially forms of *Rattus rattus* in southeast Asia is noted as a special large scale event apparently associated with deforestation and settlement

Those rats and the accompanying trombiculid mites which occur in deforested areas are not only found in greater abundance there than in the forest but they suffer local population fluctuations Both these factors abundance and considerable population increases are known to encourage evolutionary adaptions The operation of these factors may presumably be seen in the fact that taxonomists are still perplexed by the great variation shown by *Rattus rattus* and the forms of the trombiculid vector of scrub typhus Doubtless for similar reasons various strains of *Rickettsia tsutsugamushi* show considerable variation and may differ more markedly than *R prowazeki* differs from *R mooseri*

One effect of the extensive deforestation carried out by man in southeast Asia has been to encourage a joint development of large compact rat populations accompanied by a great turnover of mites potentially capable of transmitting rickettsiae It is suggested that this event has allowed a rickettsia native to the forests—either *R tsutsugamushi* or a parent form—to undergo special adaptation and development The existence of a forest enzootic rickettsiosis "jungle *tsutsugamushi*" is supported by varied evidence and there are reasonable grounds for suspecting that this jungle rickettsiosis may be widespread outside Asia

The epidemiologist needs a working hypothesis to explain the causes of concentration instead of dispersal and imbalance instead of numerical stability among living things The equatorial rain forest is an extremely stable formation developed in response to perennial heavy rainfall and high temperature Its structure is extremely complex very rich in species but with individuals of any species interspersed rather than concentrated

among plant viruses proved to be antigenic [2, 9] the first to yield large numbers of mutants experimentally [12] the first to be intensively studied chemically [7, 26] the first to be successfully identified in photographs [5, 24] and the first to be crystallized [3] In spite of all this study and much effort made to control it tobacco mosaic virus gets on well in the world and stands in no present danger of extinction

I call attention to these facts in order to compare this tobacco virus with another virus that possesses properties which are very different but which nevertheless gets along in the United States if not in the world as well or even better than tobacco mosaic virus I refer to the virus causing aster yellows the best known of the yellows type diseases Whether or not this virus occurs in large quantity in diseased plants we do not know for it has not been transmitted manually except by grafting [13] Although it has received much attention during the last 25 years aster yellows virus has not been taken to a single host plant of any species by means of juices from diseased plants Also it is one of the most unstable of all the known viruses It is so unstable that diseased plants can be cured and rendered virus free by holding them continuously at a temperature as low as 40 C for a few days [15] The thermal inactivation point of the virus is well below the thermal death points of some of its host plants such as *Nicotiana rustica* L and *Vinca rosea* L Diseased aster plants may be grown among healthy plants without any spread whatever by contact Aster yellows virus is not transmitted accidentally by man and is not ordinarily distributed by him except to a very limited extent through shipments of diseased plants Even when held at low temperatures aster yellows virus in juices from diseased plants is lost in a few days It does not retain activity in dried leaves It can infect and cause disease in more species that belong in more different families of plants than can tobacco mosaic virus [16] This however does not seem of much importance in promoting its distribution or ensuring its survival Why then is it so dynamic in its spread and so successful as a virus? Only in one respect but a very important one does it seem to have an advantage over tobacco mosaic virus It is able to multiply in a certain insect [4, 17, 18] which is an unusually efficient vector The purpose of this paper is to present evidence showing how efficiently this vector the leafhopper *Macrosteles fascifrons* Stal transmits the aster yellows virus

Materials Methods and General Plan for Securing Transmission Records

It was believed that the best way to determine the efficiency of the aster leafhopper as a vector would be to obtain transmission records for a number of individual insects This was done by allowing virus free insects to feed on a diseased plant for a given length of time and then transferring each individual to a healthy aster plant each successive day as long as it lived The insects tested were descendants of aster leafhoppers originally identifi d

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Maintenance of Yellows-type Viruses in Plant and Insect Reservoirs

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Introduction

Some virus diseases are so generally distributed and so common that *they are known to all phytopathologists The best example of such a one* ■ that caused by the virus of tobacco mosaic which is produced in large quantities by infected tobacco tomato and other commonly grown crop plants It is able to multiply more or less rapidly in cells of a large number of species belonging in some 24 different families of plants [9 10] The general distribution and high incidence of tobacco mosaic virus in fields and gardens throughout the world leads to what might appropriately be thought of as a world wide overproduction of this virus Tobacco mosaic virus is so readily transmitted manually that juice from a diseased tobacco plant diluted to one part in a million parts of water infects tobacco plants when rubbed onto their leaves Even at a dilution of one part in ten million parts of water it will infect *Nicotiana rustica* L plants Tobacco mosaic virus ■ one of the most stable of known viruses It remains viable in dried tobacco tissues for many years and in tobacco juice will survive heating at about 90° C for 10 minutes [19] Also it survives the processes to which tobacco leaves are ordinarily subjected in the manufacture of smoking and chewing tobacco [8] Every tobacco user is a potential vector Its availability high infectivity stability and wide distribution by users of cigarettes[21] and other tobacco products accounts for the role it has played in researches on funda mental problems of virology When the general suitability of tobacco mosaic virus for use in experimental work is considered it does not seem surprising that this virus has received so much attention or that it has been the object of so much study It was the first virus found to be filterable [11] the first

used All green leaves were cut from the plants before they were exposed to insects This was done to insure that no insect would be able to feed on tissues that did not show well marked symptoms of yellows The groups of insects were taken directly from rye plants to the cages containing diseased plants It was observed that some insects in each group began feeding within a few minutes after being placed in the cages and that practically all were feeding within about 15 minutes Each of the four infective feeding periods were begun about 10 00 o clock in the morning since it was found that aster leafhoppers feed very little or not at all during the night or early in the morning and late in the afternoon The insects feed almost continuously in late morning and early afternoon During the infective feeding intervals the cages containing insects were placed in a greenhouse held at about 75 F Transmission tests were carried out in the same house After exposure diseased plants were kept in other greenhouses that also were held at about 75° F The houses were fumigated at weekly intervals in order to kill any nymphs that might hatch from eggs deposited in the test plants The four groups of insects consisted of males and females in about equal numbers Because of the number of plants required it was not practicable to test the four groups simultaneously Hence the experiment was carried out during four successive years An effort was made to keep the conditions under which the tests were made as nearly comparable as possible except with regard to the lengths of the respective feeding periods The group given an infective feeding period of 2 hours was confined on a test plant on December 18 that given an infective feeding period of 1 day on February 24 that given an infective feeding period of one week on October 30 and that given an infective feeding period of 2 weeks on October 2 The insects in the different groups were tested simultaneously Because of a shortage of aster plants when insects in the third group were under test it became necessary to divide this group into two lots of 25 insects each and to begin testing the second lot 15 days after beginning a test of the first lot The insects of the second lot were transferred daily to healthy rye plants instead of to healthy aster plants Hence no record of their ability to transmit during this 15-day period was obtained The 2 hour infective feeding period group consisted of 50 insects the 1 day group of 72 insects the 1 week group of 50 insects and the 2 week group of 56 insects All tests of ability to transmit were begun immediately after insects were removed from diseased plants except in the case of the second lot in the third group as was explained above Records of tests of insects in the 2 hour and 1-day infective feeding period groups show the lengths of the incubation periods of the virus in the individuals of these groups The records of tests of insects in the other two groups do not show lengths of incubation periods of virus in individuals of these groups because the periods were obscured by long infective feeding periods

as belonging in the species *Cicadula sexnotata* (Fall)[13] in 1923 This leaf hopper was later placed in the genus *Macrosteles* and given the name *Macrosteles divisus* Uhler[7] Still later it received the name *Macrosteles fascifrons* Stal[2] the binomial under which it is now known All insects used in experiments were from vigorous cultures that seemed to be free from infection by fungi They varied from 1 to 3 days in age when taken for experimental tests All were from eggs deposited on rye plants by virus free females Care was taken not to injure insects under test The plants used were China aster *Callistephus chinensis* Nees of the variety late branching grown from seeds harvested from healthy plants set out in plots at the Rockefeller Institute for Medical Research Princeton New Jersey The plants used in tests were potted from flats to 4 inch porous clay pots and grown in a greenhouse Aster seedlings in the 5 to 9 leaf stage at time of exposure to insects were used All exposed plants that did not come down with yellows were kept under observation until they blossomed The tests were carried out in lantern globe cages The upper ends of the cages were covered with cheesecloth held tightly in place by butcher s twine The bottoms were closed by setting the cages on pieces of plate glass about ¼ inch in thickness and one foot square Before putting pots containing plants in cages they were placed in small porous clay saucers Caged plants were kept in a greenhouse while being exposed to insects No cage was opened in this house All transfers of insects were made after carrying the cages to a dimly lighted laboratory where they were opened before a window Only one cage was opened at any one time The virus used was originally obtained from yellows aster plants naturally infected in plots at the Boyce Thompson Institute for Plant Research Inc Yonkers New York Tests of ability of individual leafhoppers to transmit were begun either in fall or in winter They were made with insects in four different groups The four groups of small virus free nymphs were held on yellows aster plants for four different periods of time The periods during which the groups had access to diseased plants were the following 2 hours 1 day 1 week and 2 weeks These periods were chosen because preliminary tests had shown that a small percentage of insects would become infective when held on a diseased plant for 2 hours while a considerable number would fail to become infective when held on a diseased plant for one week The preliminary tests also had shown that some individuals in any large colony remained virus free unless they were allowed to feed on infected plants for as long as about 2 weeks

Four colonies of 10 insects each from the same sources as the insects that were tested individually were not allowed to feed on diseased plants One colony was tested with each of the four groups These served as controls

The diseased plants on which the insects were placed for their infective feeding intervals had shown yellows symptoms for about [illegible] months when

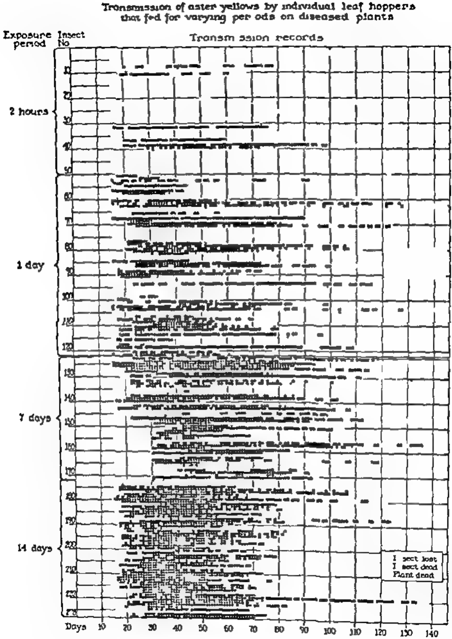
Transmission of aster yellows by individual leaf hoppers that fed for varying periods on diseased plants
Exposure period
Insect No
Transmission records
2 hours
1 day
7 days
14 days
Insect lost
Insect dead
Plant dead
Days
10
20
30
40
50
60
70
80
90
100
110
120
130
140

FIG 1

Efficiency of Macrosteles Fascifrons as a Vector of Aster Yellows

Studies on the efficiency of the aster leafhopper in spreading aster yellows disease raised several questions regarding the relationship between virus and vector Some of these were the following How long must the vector feed on a diseased plant in order to become infective? Is there a correlation between length of infective feeding period and efficiency of transmission? Is every leafhopper capable of becoming infective? Is virus retained as long as the insect lives? Do any infective individuals transmit to every plant on which they feed for as long as one day? Are some individual insects more efficient transmitters than others? Is there a falling off in efficiency of transmission with age?

Transmission records for the individual leafhoppers tested are presented in Figure 1 where black squares represent plants that became diseased while white spaces of equal size represent plants that remained healthy Since none of the plants exposed to control colonies came down with yellows these records are not included in the figure Crosses at the ends of transmission records mark the days on which insects died and were found dead in their cages Triangles mark the days on which insects that probably had died on those days could nevertheless not be found When the body could not be found it was of course impossible to be sure that the insect had died Circles in the figure represent plants that were exposed on the days indicated but died for some unknown reason before developing symptoms of yellows and before a sufficient time had passed to exclude the possibility of their having been infected Inspection of the figure will show that of the 228 insects tested only 148 transmitted aster yellows virus Eighty did not transmit Among these were 10 insects that lived for 25 days or less after their tests were started Three of these were in the 2 hour infective feeding period group 4 in the 1-day infective feeding period group and 3 in the 14 day infective feeding period group Under the conditions of the experiment 25 days was considered sufficient to give a rather good test of infectivity the usual incubation period of the virus in the insect being considerably less than 25 days Nevertheless it was not possible to say with certainty that none of the 10 some of which lived considerably less than 25 days after the tests were started carried virus or would not have transmitted virus if they had lived longer It was believed that insects which lived longer than 25 days after their tests were started would never have transmitted virus even if they had lived much longer than they did If the 10 non transmitting insects that lived 25 days or less after their tests were begun are eliminated from the experiment we see that 41 in the 2 hour group 21 in the 1-day group 8 in the 1 week group and none in the 2 week group failed to transmit Six insects in the 2 hour infective feeding period group 47 in the 1-day infective feeding period group 42 in the 1 week infective feeding period group and 53 or all in the 2 week infective

capable of becoming infective However 53 is a small number It may be that more extensive tests will show that a small percentage of leafhoppers is not capable of transmitting the aster yellows virus

The transmission records indicate that some individual insects become infective much more readily than others If it were not so all in the 7-day infective feeding period group should have become transmitters since about one-eighth of those in the 2 hour infective feeding period group transmitted It must be concluded that some individuals became infective much more readily than others

The question of whether virus is retained indefinitely by infective leafhoppers will be considered next Inspection of the Table shows that of the 148 insects that transmitted 81 or about 54 7% transmitted on the last day or the day next to the last day of their lives One hundred twenty three or about 83 1% transmitted during the last 4 days of their lives One hundred thirty six or about 91 8% transmitted during the last [illegible] days Failure of leafhoppers to infect from 5 to 10 plants consecutively and then to infect plants on which they later fed was not uncommon Eighteen insects distributed through the four groups gave a total of 30 such skips after which each of the insects again transmitted The results prove that such skips do not indicate loss of infectivity Hence insects that gave skips of such lengths immediately prior to death cannot be considered to have lost the virus or their ability to transmit Only 2 of the 148 leafhoppers that transmitted virus gave long skips just prior to death Neither was in the group that fed on diseased plants for either the short or the long period Insect no 71 in the 1-day feeding period group failed to infect plants during the last 15 days of life and insect no 132 in the 1 week feeding period group failed to infect during the last 18 days of life Insect no 132 was a poor transmitter This was shown by the fact that it carried virus to less than one half of the plants on which it fed after becoming infective and up to the time of the 18-day skip just prior to death Insect no 71 on the other hand was a good transmitter It infected 99 of the 123 plants on which it fed up to the beginning of the 15-day skip just preceding death It is not believed that the 18-day skip by insect no 132 nor the 15 day skip by insect no 71 just prior to their deaths indicates that these insects had lost ability to transmit No insect transmitted to every plant on which it fed after becoming infective but leafhopper no 68 in the 1 day infective feeding period group transmitted to 74 of the 76 plants on which it fed during its infective life One of the plants that it did not infect was exposed on the day preceding death It may be presumed that the insect was sick on that day and that it either did not feed or fed sparingly This probably accounts for its failure on that day but the other failure which occurred on the 27th day after it became infective is not explained There apparently is an element of chance in the act of transmission through feeding However insect no 68

feeding period group, transmitted The results showed that when virus free nymphs have access to a diseased plant for 2 hours about 1 in 8 become infective When they have access to such a plant for 1 and 7 days respectively about 1 in 3 and 6 in 7 become infective All insects that were allowed to feed for 2 weeks on a diseased plant and lived for 25 days or longer after the test was started became infective In experiments not reported here a few insects with infective feeding periods as short as one half hour transmitted None transmitted after an infective feeding period as short as one quarter hour The transmission records show a correlation between length of infective feeding period and efficiency of transmission

It will be seen that insect no 39 having an infective feeding period of 2 hours lived for 89 days after becoming infective and transmitted to 76 of the 89 plants on which it fed Only 1 insect of the 8 with infective feeding periods of 1 day only 4 of the 8 with infective feeding periods of 1 week and none of the 3 with infective feeding periods of 2 weeks that lived for 89 days or longer after becoming infective gave higher percentages of transmission than insect no 39 Five insects of the 19 that lived sufficiently long to be compared with no 39 gave only slightly higher percentages of transmission during comparable periods of their lives and only 1 gave a higher percentage during its entire period of infectivity

The 6 leafhoppers with 2 hour infective feeding periods fed on a total of 384 plants and transmitted virus to a total of 275 plants or to about 71% of all the plants on which they fed during the periods of their infectivity After termination of the incubation period of virus in the several insects that became infective those in the 1 day 7 day and 14-day infective feeding period groups transmitted to 72 75 and 78% of all the plants on which they fed The differences in the efficiencies of the groups as vectors are by no means proportional to the lengths of their infective feeding periods nor are they very great Nevertheless there is good evidence of a relationship between lengths of infective feeding periods and efficiency of transmission The insects that became infective by feeding on a diseased plant for from 1 to 14 days transmitted somewhat more efficiently than the insects that became diseased after access to a diseased plant for 2 hours Each of the transmitting insects in the 2 hour 1 day 7 day and 14-day groups infected an average of 45 35 39 and 40 plants respectively Thus although the transmitting insects in the 2 hour infective feeding period group transmitted to the smallest percentage of the plants on which they fed they infected on an average the largest total number of plants per insect The efficiencies of the individual insects in the different groups were remarkably similar

Judging from the records of the 53 transmitting insects in the 14-day infective feeding period group it would seem that every leafhopper is

largest numbers of plants There is no evidence as to why some insects lived so much longer than others Among the long lived insects the good transmitters infected many more plants than the poor transmitters The records give no hint as to why some were good and others poor transmitters Perhaps the virus multiplies more rapidly or reaches a higher concentration in the good transmitters than in the poor ones It is obvious that the promptness with which a newly hatched insect becomes infected determines the length of time it will be able to transmit Eggs deposited in a diseased plant produce nymphs that have the best opportunity of becoming infected early in life The length of the incubation period of the virus in the insect is considerably longer in some individuals than in others Hence length of incubation period of virus in insect is a factor of some importance The transmission records show that a few insects became infected after feeding on a diseased plant for as short a period as 2 hours while others did not become infected after feeding on a diseased plant for as long as one week Apparently some insects are infected by the virus much more readily than others although all or almost all seem susceptible to it Naturally those leafhoppers that are infected readily acquire ability to transmit sooner than those that are infected less readily The damage caused by an individual infective insect as a transmitter of yellows virus also depends on the plants to which it has access If it feeds in a rye field it will not spread yellows because rye plants are immune from this disease If it feeds in an aster field it will infect a large number of plants because the aster is highly susceptible If it feeds in a carrot or a lettuce field it will infect many plants but not so many as in the aster field because these plants although susceptible are not so highly susceptible as the aster Infective insects that are exposed to high temperatures such as frequently prevail during summer months especially in the South lose ability to transmit either temporarily or permanently depending on the length of the hot spell Thus climate and season have a profound effect on the ability of the leafhopper to transmit aster yellows virus The transmission records show that insects which acquired virus through a short infective feeding period transmitted it to a somewhat smaller percentage of plants than those that acquired virus during a long infective feeding period It may be that those that acquired virus most readily suffered some injurious effect from the infection whereas those that acquired it less readily suffered less or perhaps not at all and for this reason were better transmitters Groups with long infective feeding periods include insects that are readily infected as well as those that are infected with difficulty This obscures to some extent the differences between those that are readily infected and those that are not

Several different species of leafhoppers feed on aster plants but only one has been shown to transmit They all doubtless ingest virus as they feed but only *Macrosteles fascifrons* can transmit it and this insect apparently only if it itself becomes infected Individuals that are capable of becoming

transmitted to 98 6% of all the plants on which it fed during its infective life up to but not including the day preceding its death This is a record of high efficiency The transmission records show that some individuals were much more efficient transmitters than others

Was there a falling off in efficiency of transmission with age? Of the 6 infective insects in the group that fed for 2 hours on a diseased plant 3 lived 60 days or longer after first transmitting Of these one transmitted to more one to fewer and one to the same number of plants in the first 30 day period as they did in the second 30 day period Of the 47 infective insects in the group that fed one day on a diseased plant 13 lived 60 days or longer after first transmitting Of these 2 transmitted to more 8 to fewer and 3 to the same number of plants in the first 30 day period as they did in the second 30 day period Of the 42 infective insects in the group that fed for 7 days on a diseased plant 12 lived 60 days or longer after first transmitting Of these 9 transmitted to more and 3 to fewer plants in the first 30 day period than they did in the second 30 day period And of the 53 insects in the group that fed for 14 days on a diseased plant 9 lived 60 days or longer after first transmitting Of these 8 transmitted to more and one to fewer in the first 30 day period than they did in the second 30-day period Of the insects that transmitted in the four groups a total of 37 lived 60 days or longer after first transmitting Of these 20 transmitted to more and 17 to the same number or to fewer plants in the first 30-day period than they did in the second 30 day period The 37 insects in these four groups transmitted to 906 of the 1110 plants on which they fed in the first 30 days and to 873 of the 1110 plants on which they fed during the second 30 days They infected 81 6% of the plants in the first 30 day period and 78 6% in the second 30 day period The falling off in infectivity during the second 30 day period was slight It certainly was no more than might be expected from infective insects that were approaching old age It will be seen that most of the insects in the group that fed on a diseased plant for one day and lived for 60 days or longer after becoming infective transmitted to more plants in the second 30 day period than in the first 30 day period whereas most of the insects living 60 days or longer that fed on a diseased plant for 7 and 14 days transmitted to fewer plants in the second than in the first 30-day period

Discussion

The damage caused by an aster leafhopper in spreading yellows seems to depend chiefly on two factors—the length of life of the insect and its efficiency as a transmitter It also depends to a considerable extent on how soon it has an opportunity to feed on a diseased plant how readily it acquires virus *from such a plant what species of* plant it feeds on and what temperature prevails after it becomes infective The transmission records show that infective insects that lived the longest transmitted to the

is offered the hypothesis that length of time curly top virus is retained by the beet leafhopper depends on the size of the charge of virus acquired and this in turn [illegible]n length of infective feeding period lacks cogency

Summary

Four groups of aster leafhoppers consisting of 50 72 50 and 56 insects respectively were held on yellows aster plants for 2 hours 1 day 1 week and 2 weeks respectively The individual insects were then confined separately in lantern globe cages and allowed to feed on a different healthy young aster or rye plant each succeeding day as long as they lived A trans mission record was obtained for each of the 228 leafhoppers tested Com parable virus free aster leafhoppers that were not allowed to feed on dis eased plants served as controls Of the 50 insects in the [illegible] hour infective feeding period group 6 transmitted of the 72 in the 1-day feeding period group 47 transmitted of the 50 in the 1 week feeding period group 42 transmitted and of the 56 in the 2 week feeding period group 53 transmit ted None of the other 80 insects in the four groups and none of the control insects transmitted Among the 80 leafhoppers in the four groups that did not transmit 10 lived for 25 days or less after the tests were started This period was considered too short to be satisfactory One or more of the 10 might have transmitted if it had lived longer Of the other 70 insects that did not transmit, 41 were in the 2 hour feeding period group 21 in the 1-day feeding period group and 8 in the 1 week feeding period group All of the 53 insects in the 2 week feeding period group that lived for 25 days or longer after the tests were started transmitted After a 2 hour infective feeding period about 1 in each 8 insects transmitted while after a 1-day and a 1 week feeding period about 3 in each 4 and about 6 in each 7 transmitted The records show that although some insects became infected during a 2 hour infective feeding period others did not become infected during a 1 week infective feeding period The results indicate that although most if not all individuals are susceptible to infection by the virus some are much more susceptible than others A considerable number of insects remain infective as long as they live Although there is no proof that all infective individuals remained infective to the ends of their lives the records bring no evidence to the contrary The insects with long infective feeding periods transmitted to slightly higher percentages of plants than those with short infective feeding periods The records show that after becoming infective the insects transmitted virus to about three fourths of all the plants on which they fed during the remainder of their lives The aster leafhopper [illegible] an efficient transmitter and a superb reservoir of the virus

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infective may live on a diseased plant for a period approaching 2 weeks without becoming transmitters

As was stated in the introduction to this report aster yellows spreads and flourishes in this country at least as well as tobacco mosaic This is true in spite of the fact that it cannot be transmitted to plants manually except by grafting does not spread by contact is so unstable as to be inactivated *in vivo* by relatively low temperatures and generally is not distributed by man The secret of its great success as a virus rests on an ability that more than compensates for these deficiencies It is able to infect and multiply in an insect that feeds on many different species of plants including the aster It has now been shown that a single infective individual feeding for one day will transmit to about three of every four aster plants on which it feeds Aster yellows virus like many others lives overwinter in biennial and perennial plants that serve as its reservoir out of doors and on annual biennial and perennial plants indoors during the winter season of the year It is maintained out of doors during spring summer and fall in annual as well as in biennial and perennial plants Its spread to plants is exclusively or almost exclusively by the aster leafhopper Leafhopper vectors of other yellows type viruses doubtless play an equally important role

It has been claimed that the virus of curly top of sugar beets[5,6] transmitted by the beet leafhopper and the virus of sugar beet yellows [7] transmitted by the peach aphid are retained for a long time by these insects when the latter are allowed to become viruliferous through feeding on diseased beets for a long time but are soon lost when the vectors are allowed to become viruliferous through feeding on diseased beets for short periods The correlation between lengths of infective feeding periods on diseased beets (presumably the amounts of virus taken up) and lengths of virus retention periods has been presented as evidence that curly top and beet yellows viruses do not multiply in their vectors In the case of the aster leafhopper as transmission records show there is no correlation between length of infective feeding period and length of time virus is retained by the leafhoppers This is exactly what might be expected if as is now known the aster yellows virus multiplies in the aster leafhopper In one respect the beet leafhopper and the aster leafhopper behave similarly toward the viruses they transmit Even after relatively long infective feeding periods some individuals of both species fail to become viruliferous This is readily explained in the case of the aster leafhopper by assuming that some individuals are much less susceptible to infection by aster yellows virus than are others If beet leafhoppers having a short infective feeding period take up a small charge of virus and those having a long infective feeding period take up a large charge it is difficult to understand why some individual leafhoppers that would become viruliferous if allowed a very long infective feeding period acquire no virus at all during an interval as long as 8 days on a diseased plant Until some satisfactory explanation of this behavior

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Summary

Four groups of aster leafhoppers consisting of 50 72 50 and 56 insects respectively were held on yellows aster plants for 2 hours 1 day 1 week and 2 weeks *respectively* The individual insects were then confined separately in lantern globe cages and allowed to feed on a different healthy young aster or rye plant each succeeding day as long as they lived A transmission record was obtained for each of the 228 leafhoppers tested Comparable virus free aster leafhoppers that were not allowed to feed on diseased plants served as controls Of the 50 insects in the 2 hour infective feeding period group 6 transmitted of the 72 in the 1-day feeding period group 47 transmitted of the 50 in the 1 week feeding period group 42 transmitted and of the 56 in the 2 week feeding period group 53 transmitted None of the other 80 insects in the four groups and none of the control insects transmitted Among the 80 leafhoppers in the four groups that did not transmit 10 lived for 25 days or less after the tests were started This period was considered too short to be satisfactory One or more of the 10 might have transmitted if it had lived longer Of the other 70 insects that did not transmit 41 were in the 2 hour feeding period group 21 in the 1-day feeding period group and 8 in the 1 week feeding period group All of the 53 insects in the 2 week feeding period group that lived for 25 days or longer after the tests were started transmitted After a 2 hour infective feeding period about 1 in each 8 insects transmitted while after a 1-day and a 1 week feeding period about 3 in each 4 and about 6 in each 7 transmitted The records show that although some insects became infected during a 2 hour infective feeding period others did not become infected during a 1 week infective feeding period The results indicate that although most if not all individuals are susceptible to infection by the virus some are much more susceptible than others A considerable number of insects remain infective as long as they live Although there is no proof that all infective individuals remained infective to the ends of their lives the records bring no evidence to the contrary The insects with long infective feeding periods transmitted to slightly higher percentages of plants than those with short infective feeding periods The records show that after becoming infective the insects transmitted virus to about three fourths of all the plants on which they fed during the remainder of their lives The aster leafhopper is an efficient transmitter and a superb reservoir of the virus

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16

Variation in Virulence of "Rickettsia Rickettsii" under Natural and Experimental Conditions*, †

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For the past three years studies have been carried out mainly in Mary land and Montana on various aspects of the natural history of Rocky Mountain spotted fever (RMSF) The results of some of these experiments will be reported in this paper

For those readers wishing a knowledge of earlier investigations on the natural history of RMSF the papers of Ricketts[1] should be consulted This work done over forty years ago stands as a landmark in this field and *indeed is one of the most brilliant pieces of research that has ever been* carried out on the natural history of an infectious agent The classical paper of Wolbach may also be consulted as well as the reports of Spencer and Parker[3] Parker[4] and Topping[5]

The problems of studying the variation in virulence of a parasite are manifold For example one wonders whether the biological characteristics

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† Some of the experiments described in this paper will be reported in the American Journal of Hygiene in greater detail

‡ I wish to express my sincere thanks to Dr Carl L Larson Director of the Rocky Mountain Laboratory National Microbiological Institute Hamilton Montana for placing the facilities of the laboratory at my disposal Some of the experiments reported in this paper were carried out at that laboratory
This work could not have been carried out without the valuable assistance of Mr C H Sawyer Miss Hope Emerson Miss Claire Preston Mr James Johnson Mr Richard Layton Mr Edgar Pickens Mr Harley Sargent and Mr Lawrence Humble I also wish to thank Dr David Lackman for performing some of the complement fixation tests during the early part of the work

of the organism isolated by laboratory procedures are really the same as they were in nature Another problem that arises is the question of the homogeneity of the isolated strain Thirdly there is the problem of interpreting the behavior of the organism in nature in terms of laboratory experiments Examples of all these problems will be illustrated in the following discussion

The paper has been divided into three parts The first section is concerned with the effect of the environment on the virulence of *R rickettsii* the causative agent of RMSF The second part deals with the reactivation phenomenon of *R rickettsii* In the third section the effect of animal and arthropod passage on the virulence of *R rickettsii* will be discussed

The Four Representative Strains of "R rickettsii"

Our results indicate that at least four different representative strains of *R rickettsii* can be distinguished in the United States The properties on

Table 1

VARIATION IN PROPERTIES FOR GUINEA PIGS OF THE FOUR REPRESENTATIVE STRAINS OF *R rickettsii* INOCULATED INTRAPERITONEALLY WITH THE SAME NUMBER OF INFECTIVE ORGANISMS (10 000 EGG LD_{50} (= 10 000 GUINEA PIG ID_{50}))

Strain	*Average days of fever*	*Average height of fever*	*Scrotal reaction*	*Per cent fatality*	*Incubation period (days)*	*Persistence in animals (days)*
R	8 1 ± 1 3	40 6 ± 12	++++	33	2	32
S	4 1 ± 65	40 4 ± 11	++	0	2	26
T	4 0 ± 92	40 3 ± 15	0	0	2	26
U	0	0	0	0		10

Particular lots of guinea pigs differ in their susceptibility to the various strains of *R rickettsii* especially in their reaction to the lower virulent strains It is therefore essential when testing unknown isolations for their virulence to run standard controls of the various strains of *R rickettsii* This has been done in all of the work reported in this paper In those guinea pigs showing lower susceptibility to the less virulent strains of *R rickettsii* the animals show a day longer incubation period than animals inoculated with the R strains

which the classification is based are shown in Table 1 It should be mentioned that the properties of the strains of *R rickettsii* shown in Table 1 are relatively independent of the numbers of organisms injected into the animals[8] over a range varying from 100 000 ID_{50} to 10 ID_{50} It will be shown later that these strains probably exist in nature since the interference phenomenon involved in the isolation by animal inoculation greatly reduces the possibility of selecting mutant strains in the laboratory which do not possess all of the biological properties of the organism as it existed in nature

Strains R S T and U have been isolated from *D andersoni* and strains R T and U from *D variabilis* Strains U and S have been isolated from

Haemaphysalis leporis palustris (however see later discussion) Only R or T representative strains have been recovered from human infections The R strain is found mainly in the Rocky Mountain area while the T strain is found principally in the eastern part of the United States * The U strain of which there may be at least two different kinds exists both east and west of the Mississippi River It may be noted here that although there are differences between the T and R strains insofar as pathogenicity for the guinea pig is concerned both may have the same pathogenicity for humans since the age specific fatality rate and clinical symptoms in humans caused by *R rickettsii* in the eastern and western part of the United States is similar[8] Indeed two T strain isolations have been made from two fatal human cases of Rocky Mountain spotted fever

Table 2

MINIMUM NUMBER OF 4 STRAINS OF *R rickettsii* REQUIRED TO INFECT AND KILL EGGS AND TO INFECT GUINEA PIGS

Minimal number of rickettsiae to		
Infect		*Give 1 LD_{50} for eggs*
Eggs	*Guinea pigs (ID)*	
R 1–10	20–100	20–100
S 1–10	20–100	20–100
T 1–10	20–100	20–100
U 1–10		20–100

THE PRODUCTION OF TOXIN AND HEMOLYTIC FACTORS BY STRAINS OF *R rickettsii*

Strains	*Toxin (mouse LD_{50})**	*Hemolytic unit*
R	1 LD (7 7 $\times$ 10)†	1 unit (1 9 $\times$ 10^{10})†
S	1 LD (1 1 $\times$ 10^{8})	1 unit (2 6 $\times$ 10^{3})
T	1 LD (9 7 $\times$ 10)	1 unit (2 1 $\times$ 10^{10})
U	1 LD (1 4 $\times$ 10^{8})	1 unit (3 1 $\times$ 10)

* These figures are lower than those reported previously since the previous work was done with rickettsiae which had been frozen for some time The above experiments were carried out with freshly harvested material

† Number of rickettsiae contributing 1 mouse LD_{50} or 1 hemolytic unit These values are the mean values taken from 3 experiments with the average error of the mean being ± 20%

Table 2 shows some further properties of the four representative strains of *R rickettsii* The organisms were prepared and counted under the electron microscope as described previously with the average error of the mean

* These facts had been shown by previous investigators Our work simply confirms them

being ± 8% for the counting method[6] It will be seen from Table 2 that the animals inoculated in Table 1 received the same number of organisms within a factor of about three this error being based on the infectivity tests The tests were carried out as described previously[6]

Although the four strains are very similar antigenically they can be distinguished immunologically by infecting guinea pigs with small numbers of ether killed rickettsiae as described in an earlier report[6] A typical experiment is shown in Table 3 The vaccinated guinea pigs are more resistant to challenge with the homologous strain than they are to challenge with a heterologous strain

Table 3

ANTIGENIC DIFFERENCES INDICATED BY RESPONSE OF VACCINATED GUINEA PIGS TO CHALLENGE BY RICKETTSIAE OF THE SAME STRAIN AS COMPARED TO OTHER STRAINS*

Challenged with strain	*Guinea pigs vaccinated with strain*			
	R(1×10^6)†	S(1×10^6)†	T(1×10^6)†	U(1×10^6)†
R	4/20	16/20	15/20	17/20
S	10/20	3/20	12/20	14/20
T	16/20	14/20	4/20	16/20

* All guinea pigs were challenged with 10 ID_{50} prepared from yolk sac material 1 week after two injections of vaccine the latter being one week apart

† Number of rickettsiae injected into guinea pig

Numerator equals number of guinea pigs showing clinical infection. Denominator shows number of guinea pigs challenged

Variation in Virulence by Locality

One of the most interesting features of the natural history of RMSF is that there appears to exist in nature localities of high and low virulence insofar as the human host is concerned which remain relatively constant year after year This observation was first made by Ricketts[1] and later by Parker[4] We have extended these observations by studying the strains of *R rickettsii* recovered from ticks in these infected localities These studies have been carried out most extensively on the east and west sides of the Bitter Root Valley in Montana Two localities were selected on each side and *D andersoni* adult ticks collected each of three years This region was chosen since although the east side is separated from the west side by only seven miles and many tick infested animals have crossed back and forth between both sides for many years the west side has had many severe cases of RMSF over the years while no case of Rocky Mountain spotted fever has ever been definitely proven to have occurred on the east side of the valley Table 4 shows a summary of the isolations made from adult *D andersoni* ticks on the east and west side of the Bitter Root Valley during the past three years Before testing all ticks were given a blood meal so as

Haemaphysalis leporis palustris (however see later discussion) Only R or T representative strains have been recovered from human infections The R strain is found mainly in the Rocky Mountain area while the T strain is found principally *in the eastern part of the United States* * *The U strain* of which there may be at least two different kinds exists both east and west of the Mississippi River It may be noted here that although there are differences between the T and R strains insofar as pathogenicity for the guinea pig is concerned both may have the same pathogenicity for humans since the age specific fatality rate and clinical symptoms in humans caused by *R rickettsii* in the eastern and western part of the United States is similar [5] Indeed two T strain isolations have been made from two fatal human cases of Rocky Mountain spotted fever

Table 2

MINIMUM NUMBER OF 4 STRAINS OF *R rickettsii* REQUIRED TO INFECT AND KILL EGGS AND TO INFECT GUINEA PIGS

Minimal number of rickettsiae to		
Infect		*Give 1 LD for eggs*
Eggs	*Guinea pigs (ID)*	
R 1–10	20–100	20–100
S 1–10	20–100	20–100
T 1–10	20–100	20–100
U 1–10		20–100

THE PRODUCTION OF TOXIN AND HEMOLYTIC FACTORS BY STRAINS OF *R rickettsii*

Strains	*Toxin (mouse LD)*	*Hemolytic unit*
R	1 LD (7 7 × 10)†	1 unit (1 9 × 10)†
S	1 LD (1 1 × 10^{8})	1 unit (2 6 × 10)
T	1 LD (9 7 × 10)	1 unit (2 1 × 10)
U	1 LD (1 4 × 10^{8})	1 unit (3 1 × 10^{20})

* These figures are lower than those reported previously since the previous work was done with rickettsiae which had been frozen for some time The above experiments were carried out with freshly harvested material

† Number of rickettsiae contributing 1 mouse LD_{50} or 1 hemolytic unit These values are the mean values taken from 3 experiments with the average error of the mean being ± 20%

Table 2 shows some further properties of the four representative strains of *R rickettsii* The organisms were prepared and counted under the electron microscope as described previously with the average error of the mean

* *These facts had been shown by previous investigators Our work simply confirms them*

on one side of the valley to determine the infection rate also appears to be acting on the other side

Although there are ecological differences between the two sides of the valley it has been impossible as yet to determine why no high virulent strains have not been found on the east side of the valley It might be mentioned however that ticks on the east side which in the laboratory are infected with virulent strains can transmit the infection to ground squirrels and chipmunks as readily as ticks found on the west side Furthermore there is no difference in the per cent of transovarial transmission between ticks collected from both sides of the valley when both are infected with virulent strains

When one considers the biological survival mechanism of *R rickettsii* the above observations are quite remarkable It was established by the brilliant work of Ricketts[1] that there are probably two biological survival mechanisms for *R rickettsii* In simplified form they may be stated as follows The organism winters over in a nymph or adult tick In the spring the infected tick bites an animal This animal is then able to infect uninfected ticks which are feeding on it The second survival mechanism is transovarial transmission in which an infected female engorges in the spring lays eggs and the eggs contain the rickettsiae The parasite is then retained by the tick throughout its larval nymphal and adult stages A great many studies which have been carried out with several strains of *R rickettsii* and several lots of *D andersoni* indicate that in the field the transovarial transmission takes place about 30% of the time Even when it does occur rickettsiae are not passed to all the eggs This mechanism by itself would appear to be insufficient to maintain the disease in nature and it appears that both of the above mechanisms tend to keep the disease going in nature

In the light of the above results it is extremely difficult to understand why both sides of the valley show a similar trend in the infection rate in the same year and why the R to U ratio on the west side remains relatively constant year after year since there would seem to be so many places in the biological survival cycle of the parasite for differences to occur in the population of ticks infected

Isolations carried out in a locality in Maryland over the three year period have yielded only U strains with the total number of infected ticks remaining approximately at 0 3% during this interval

Recently we have been able to show a tick to tick transmission of U type strains isolated from *D variabilis* in the East using ground squirrels and chipmunks for the intermediate animal host and larva and nymphs of *D andersoni* as the insect vector thus approximating the natural conditions However the proportion of uninfected ticks that were infected by feeding on the animals on which the infected ticks were also feeding was much smaller than that found when similar experiments were carried out with

to ensure that the rickettsiae would be in their most virulent phase (see later discussion) Furthermore, in all isolations the original tick suspensions were titrated in eggs as well as being inoculated into guinea pigs intra peritoneally *twenty animals being used for each isolation* Thus all isola tions described in Table 4 and indeed all of the isolations which we have made during this work are compared on a quantitatively equal basis *

Table 4

ISOLATIONS OF *R rickettsii* FROM *D andersoni* ON THE EAST AND WEST SIDES OF THE BITTER ROOT VALLEY

Year	Type	Number of isolations	Number of ticks tested	Per cent positive groups	U/R
East side					
1951	U	10	512	1 95	
1952	U	2	632	316	
1953	U	1	321	312	
West side					
1951	R	7			
	S	1			
	T	3			
	U	24	1125	3 11	3 4
1952	R	2			
	U	7	1291	0 697	3 5
1953	R	1			
	U	3	512	0 781	3 0

Ticks were tested in groups of 4–5 Thus while the above values are minimum number of isolations due to relatively few numbers of infected ticks the above calculations give values that are probably representative of the situation

Three tentative inferences can be drawn from Table 4 In the first place only low virulent strains exist on the east side while a combination of low and high virulent strains exist on the west side Secondly the ratio of the R to U strain on the west side remains fairly constant from year to year Un fortunately the summer of 1953 was very hot and it was not possible to collect very many ticks Admittedly more isolations are needed during sub sequent years before we can be definitely certain that the ratio of R to U strains is relatively constant on the west side Thirdly the fraction of the to tal ticks that are infected in nature appears to vary widely from year to year with both sides of the valley showing corresponding trends in the same year The reason for this varied infection rate of arthropod hosts is not known but does not appear to be related to the variation in the size of the tick popu lation in the localities studied Whatever environmental factor is operating

* Tests for the *homogeneity of all isolations made during our three year study were* carried out as described previously

through many generations in the laboratory and were free of *R rickettsii* were put on each of the three squirrels The uninfected nymphs were allowed to engorge and molt to adults After one month at room temperature 30 adults from each squirrel were put on each of two guinea pigs The adults were allowed to feed for 6 days and the animals then observed for three weeks In control experiments the low virulent and high virulent infected samples were set up exactly as described above except that group 1 had no high virulent infected ticks and group 2 no low virulent infected ticks

Table 5

INTERFERENCE BETWEEN INFECTIONS WITH A STRAIN OF LOW AND ONE OF HIGH VIRULENCE OF *R rickettsii* AS DEMONSTRATED IN GROUND SQUIRRELS INFECTED BY MEANS OF *D andersoni* TICKS

Normal ticks fed on squirrels infected by exposure to	*Reactions of guinea pigs on which normal ticks fed after feeding on infected squirrels*		
	Average days of fever	*Scrotal reaction*	*Fatality*
(1) Low virulent infected ticks	5 1 ± 93	0/20	†0/20
(2) Highly " "	8 2 ± 1 4	17/21	8/ 1
(3) Low and highly " "	4 6 ± 81	0/19	0/19

Numerator shows number of animals showing scrotal reaction Four animals in group (1) showed no symptoms 3 animals in group (2) showed no symptoms and 5 animals in group (3) showed no symptoms Fever equals any temperature over 39 8 C All scrotal reactions were severe resulting in necrosis in group (2)

† Numerator shows number of animals dying of spotted fever

The results shown in Table 5 are the cumulative data from four experiments all experiments giving the same results It can be readily seen that uninfected ticks feeding on Columbian ground squirrels harboring a large number of *D andersoni* infected with low virulent strains and a small number of *D andersoni* infected with a highly virulent strain of *R rickettsii* transmit only a low virulent strain It is not possible to say from these experiments whether interference between the high and low virulent strains occurs in the ticks All tests carried out so far indicate that such ticks contain only low virulent strain Larvae coming from female of such ticks have been found to have the same strain as the parent Experiments similar to the above were carried out with field mice (Microtus) and cotton tail rabbits with similar results In other experiments in which ticks harboring highly virulent strains predominated normal ticks became infected only with the highly virulent strain Larvae coming from females of such ticks have been found to have the same strain as the parent Thus it is easy to see why if one strain is predominant in an area it will stay predominant However the importance of the role of the interference phenomenon in the natural

virulent strains The tests for infectivity were carried out by inoculating chick embryos and making two blind egg passages as described previously[6] The per cent transovarial transmission rate of the eastern U strains appears to be similar to that found for the virulent strains These results must be considered as preliminary and further studies which are in progress must be completed before any definite statement can be made as to the relative ease of infecting ticks with the eastern U strains under natural conditions All that can be said at the present time is that the transmission of U type strains is possible under conditions closely approximating those found in the field

Under conditions similar to those used in the above experiments it has so far been impossible to show tick to tick transmission with the U strains isolated from *D andersoni* in the West Columbian groundsquirrels chipmunks field mice (Microtus) and cotton tail rabbits have been used as the animal host in these experiments Further studies are in progress on the transmission of these strains

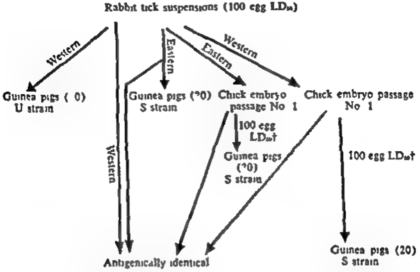
Another difference between the U strains found in the eastern part of the United States as compared to those found in the West is that the eastern U strains after 1 or 2 egg passages will behave like T strains when inoculated into guinea pigs The U strains isolated in the western part of the United States remain U strains even after 10 egg passages These results have been consistent for eight eastern U strains and twelve western U strains All these experiments were carried on a quantitatively equal basis and the original strains isolated directly from the various tick species were used in all trans mission studies

The eastern U strain can also be distinguished from the U strain found in the western part of the United States by tissue culture methods Using human testicular tissue the eastern U strain behaves like a T strain after one tissue culture passage It also causes a cytopathogenic effect in the cells Indeed tissue cultures infected with these strains grow very poorly The virulence of the western U strains has not been changed under similar con ditions

One factor which may play a role in determining the kind of strain found in a locality is the interference phenomenon We have found that if two strains are inoculated intraperitoneally simultaneously into guinea pigs the one which is in the greatest number will inhibit the multiplication of the other strain[7] We have extended these observations in an attempt to deter mine if the interference phenomenon[7] would play a role in maintaining areas of essentially pure strains of *R rickettsii* in nature The following type of experiment was carried out as shown in Table 5 One hundred *D andersoni* nymphs infected with the low virulent strain T and two *D andersoni* nymphs infected with the highly virulent strain R were put on each of three Columbian ground squirrels After three days during which time 80% of the nymphs infected with the T strain and both nymphs infected with the R strain had been feeding 100 uninfected nymphs which had been raised

Table 7

A COMPARISON OF THE PROPERTIES OF *R rickettsii* ISOLATED FROM *Haemaphysalis leporis palustris* COLLECTED EAST AND WEST OF THE MISSISSIPPI RIVER



* Titer of original tick suspensions per 0 5 ml of suspension 0 5 ml of these suspensions was inoculated intraperitoneally into guinea pigs 20 animals being used for each suspension

† Dose inoculated into guinea pigs as determined by egg titration

of the Mississippi This table is based on eight eastern isolations and twelve western isolations The strains isolated east of the Mississippi are of the S type All of the strains isolated west of the Mississippi appear to be of the U type After one egg passage however they all show the S strain characteristics Immunologically both isolations are identical when tested by the sensitive method described earlier in this paper The eastern isolations of *R rickettsii* may be passed as many as ten times in chick embryos and they still give the characteristic S strain response in guinea pigs

The Reactivation Phenomenon

It was found by Spencer and Parker[3] that if *D andersoni* ticks infected with a virulent strain of *R rickettsii* were refrigerated for several months and then made into suspension and inoculated into guinea pigs the guinea pigs showed at most only a few days of fever and were immune to a virulent challenge of *R rickettsii* It has been thought that this was due to the fact that the tick suspensions contained too few organisms to infect but enough to immunize However by egg titrations of such suspensions we have been able to show that such is not the case Table 8 shows a comparison of the

history of *R rickettsii* would depend upon many factors such as the number of susceptible animals available in an area and the number of infected ticks It is therefore impossible to assess the importance of this phenomenon at the present time However it is obvious that the interference phenomenon would greatly limit the development of new strains in nature by selection and mutation since even the U strain which does not multiply in animals will inhibit the multiplication of the R type when present in 10 times the concentration of the R strain Thus if mutants arose at the rate of 1 in 10^5 or 10^8 organisms as they do with other microorganisms then multiplication would be inhibited and there would be very little chance for their development

Another effect of environment on *R rickettsii* appears when one compares the isolation of *R rickettsii* from *Haemaphysalis leporis palustris* (H lp) found east and west of the Mississippi When strains are isolated from H lp in nature they have always been of low virulence [4] We have confirmed and extended these observations Table 6 shows the characteristics of the rabbit tick isolations found in the west It can be readily seen that the isolations from the tick behave like a U strain After one egg passage however it behaves like the S strain On looking at Table 7 it will be seen that there is a curious difference in the strains isolated east and west

Table 6

PROPERTIES OF *R rickettsii* ISOLATED FROM *Haemaphysalis leporis palustris* FOUND IN THE BITTER ROOT VALLEY‡

RABBIT TICK SUSPENSION FROM NATURE COLLECTED FROM COTTON TAIL RABBIT

1 000 LD *

↙	↓ Egg passage No 1 ↓ 1 000 LD †
Guinea pigs (30)	*Pigs (30)*
1) Slight if any symptoms	1) Scrotal reaction resulting in swelling and redness and several days of fever
2) Low CF	2) High CF
3) Immune to virulent S F	3) Immune to S F and boutonneuse
4) Not immune to boutonneuse	4) Titer of spleen 10–100 LD
5) Titer of spleen 0 LD_{50}	5) Can be passed serially in guinea pigs
6) Cannot be passed serially in guinea pigs	

* Titer of original tick suspension per 0 5 ml inoculated intraperitoneally into 30 guinea pigs

† Titer of material per 0.5 ml inoculated intraperitoneally into guinea pigs

‡ Similar strains have been isolated from this rabbit tick in other western states

the virulent state thus appears to be hereditary since the avirulent phase can not be passed serially from guinea pig to guinea pig Indeed all attempts to demonstrate multiplication in guinea pigs of the avirulent phase prepared from refrigerated tick suspensions have been completely negative These at tempts have included titrations in chick embryos of various organs of guinea pigs inoculated with 100 egg LD_{50} of the avirulent phase and also passage of these organs to other guinea pigs at two day intervals for 14 days None of these guinea pigs injected with the various organs showed any RMSF symptoms and none were immune to challenge with 5 ID_{50} of a virulent strain of *R rickettsii* As many as three blind passages in guinea pigs have been carried out in an attempt to demonstrate multiplication of the avirulent phase with entirely negative results Similar negative experiments have been carried out with Columbian ground squirrels cotton tail rabbits chipmunks and cynamologous monkeys It would appear that when the avirulent phase from refrigerated ticks is injected into animals one is essentially vaccinating the animals since in practically all respects such as the clinical response and immunological response the avirulent phase behaves like a killed vaccine prepared from the highly virulent phase

The relationship of avirulent phases to masked viruses and the im portance of possible avirulent phases in the study of the natural history of an infectious agent has been discussed in a previous article [8] One also wonders whether the avirulent phase of *R rickettsii* is in any way related to an incomplete rickettsia in the sense that incomplete forms exist for viruses [9] Experiments are in progress on this latter point

Table 9

THE EFFECT OF MOLTING ON THE VIRULENCE OF *R rickettsii* IN *D andersoni*

Time assayed after engorgement	*Egg LD_{50} per 5 ticks*	*Reaction in guinea pigs*
0	10 000	Virulent spotted fever
1 week	100 000	Virulent spotted fever
2 weeks	30 000	Virulent spotted fever
3 weeks	30 000	Virulent spotted fever
4 weeks (just before molting)	10 000	Virulent spotted fever
5 weeks (just after molting)	10 000	Mild spotted fever
7 weeks (2 weeks after molting)	2 000	Mild spotted fever

30 animals injected in each time period Virulent spotted fever results in 8 days of fever severe scrotal reactions resulting in the death of about 1/3 of the animals 10 LD_{50} at any time during 4 weeks also gave similar reaction Mild spotted fever means only 3 to 4 days of fever and no other symptoms

A further analysis of this phenomenon has shown that the main decrease in virulence takes place immediately after molting This is shown in Table 9 Nymphs of *D andersoni* were infected with the virulent R type by feeding on infected guinea pigs They were allowed to engorge and fall off the ani mal which took about one week The ticks were then kept at room temper

Table 8

A COMPARISON OF THE VIRULENT AND AVIRULENT PHASES OF *R rickettsii* FOR THE GUINEA PIG*

Avirulent phase	*Virulent phase*
Grows poorly (0 LD_{50} in spleen)	Grows well (300 LD_{50} in spleen)
Produces low if any complement fixation titer (0–1 20)	Produces high complement fixation titer (1 254–1 1024)
Produces toxin neutralizing antibody	Produces toxin neutralizing antibody
Cannot be passed serially from pig to pig	Can be passed from pig to pig
Produces immunity to virulent spotted fever	Produces immunity to virulent spotted fever
Produces no immunity to boutonneuse fever	Produces immunity to boutonneuse fever
Changed to highly virulent form by 1 egg passage	Virulence not changed by 1 egg passage
Changed to highly virulent state by keeping ticks infected at 37 C for 72 hours	Virulence not changed by keeping ticks infected with virulent form at 37 C for 72 hours
Produces little if any fever	Produces about 8 days of fever
Produces no scrotal reaction	Produces severe scrotal reaction
No fatality of guinea pigs	25 to 50% fatality

Table based on 100 guinea pigs injected with 100 egg LD_{50} of avirulent phase and 100 guinea pigs injected with egg LD_{50} of virulent phase The avirulent phase was prepared from refrigerated ticks as described in an earlier report

avirulent and virulent phase of *R rickettsii* This chart is based on intraperitoneal inoculation of 100 guinea pigs for each phase 100 egg LD_{50} being injected for each phase Thus the guinea pigs inoculated with the avirulent phase were receiving 100 times the number of organisms necessary to cause a virulent spotted fever infection since 1 egg LD_{50} will cause a virulent spotted fever infection The avirulent phase can be made virulent by a blood meal or by keeping the ticks at 37 C for 48 hours [8] We have confirmed these results Furthermore we have found that the change from the avirulent to the virulent phase may also be brought about by one chick embryo passage This is of extreme importance since it permits us to count the rickettsiae of the avirulent phase by egg titration

Other experiments have indicated that the reactivation phenomenon is not due to selection spontaneous mutation or interference If an inhibitor is present in the avirulent phase tick suspensions which prevents the guinea pigs from coming down with spotted fever experiments have shown that it is never in excess over that required to neutralize the possible virulent organisms that might be present and it must be closely bound to the rickettsiae All data indicate that a change occurs which makes the virulent organism avirulent and that under appropriate conditions the organism can be made virulent again Once reactivation occurs the rickettsiae can be transferred by spleen passage from guinea pig to guinea pig for at least 16 transfers without changing its biological properties The change from the avirulent to

Since the R S and T strains of spotted fever have a virulent and aviru lent phase this brings up the question of whether the S T and U strains are not simply different virulent phases of the R strain The evidence against this is as follows (1) the R S T and U strains have been maintained in eggs for many passages (one egg passage will reactivate the avirulent phase) (2) while the R S T and U strain can be distinguished immunologically from each other the avirulent and virulent phases of any one strain cannot be distinguished (3) the S and T types have been carried through many arthropod animal passages and still retain their characteristics One blood meal in ticks will reactivate the avirulent phase (4) ticks infected with the R type have been kept for as long as three years in the avirulent phase and when reactivated give the characteristic virulent R strain The available data indicate that this is about the longest time that adult ticks can exist in nature without feeding on an animal and therefore this would be the longest time the avirulent phase could exist in nature

There is no evidence from field observations that the S T and U strains are simply variants of the R strain Areas have been found where only the T or U strains are present While all of this evidence does not rule out the fact that the four strains of rickettsiae may come from one strain or are different virulent phases of one strain it is difficult to fit all the evidence into such a hypothesis

The Effect of Animal and Arthropod Passage on the Virulence of "R rickettsii"

Since several different virulent strains of *R rickettsii* are found in nature experiments have been carried out to see whether mechanisms exist in nature for selecting strains of a particular virulence or whether they arise by a random process

In the first part of this section the effect of animal passage on the viru lence of *R rickettsii* will be discussed

Strains of *R rickettsii* are always of low virulence when isolated from *Haemaphysalis leporis palustris* the rabbit tick * Since the rabbit tick only feeds on rabbits and birds and in the areas we have studied it seems to feed only on rabbits it was possible that the low virulence of *R rickettsii* isolated from *Haemaphysalis leporis palustris* was due to its continued passage in rabbits Therefore virulent R strains were passed by spleen transfer in cotton tail rabbits Table 10 shows the result After 8–11 transfers the strains always showed the virulence of S strains and in one instance a T strain Continued spleen passage in dogs and opossums also lowered the virulence of the R strains No decrease in virulence was noted in passing R strains in Columbian ground squirrels chipmunks and field mice However if instead of using spleen passage the virulent R strains are passed from

* This observation was first made by R R Parker on rabbit ticks collected in the Bitter Root Valley back in the 1920 s

ature Samples of five ticks were then assayed in eggs and inoculated intraperitoneally into twenty guinea pigs at the intervals shown in Table 9 The LD_{50} refers to the titration of the tick suspension in eggs per 0 5 ml of suspension *0 5 ml of each suspension was inoculated intraperitoneally into* the guinea pigs Up to the time that nymphs molt into adults they have the virulent strain However as soon as they molt the adults no longer have the virulent strain but give a type of reaction similar to the T type in spite of the fact that about 10 000 times the number of organisms were injected into the animals than is necessary to give a virulent reaction The same decrease in virulence cannot be shown when larvae infected with the virulent strain molt into nymphs These experiments have been repeated four times with essentially the same results This result brings up the very interesting question whether the molting hormone of the tick can control the virulence of the organism The avirulent phase found after molting however appears to differ from that found in refrigerated ticks in that if a spleen suspension prepared from a guinea pig inoculated with a suspension of recently molted infected ticks is injected into another guinea pig that guinea pig comes down with virulent spotted fever This result is independent of whether the guinea pig is injected with 2 egg LD_{50} or 10 000 egg LD_{50} of the avirulent phase The avirulent phase prepared from recently molted ticks shown in Table 9 can also be shown to multiply in guinea pigs It is not clear therefore whether the avirulent phase prepared from recently molted ticks kept at room temperature is of a slightly different kind than that prepared from refrigerated ticks or whether there are still some virulent organisms present in the recently molted ticks kept at room temperature which overgrow the avirulent phase on the second guinea pig passage In view of the interference phenomenon however it is difficult to see how it could be due to the latter hypothesis

The S and T strains of spotted fever have also been found to show the reactivation phenomenon that has been described above for the R strain That is the S and T strains show avirulent and virulent phases The avirulent phase of the S and T strains can be reactivated by the same procedures as described above for the R strains

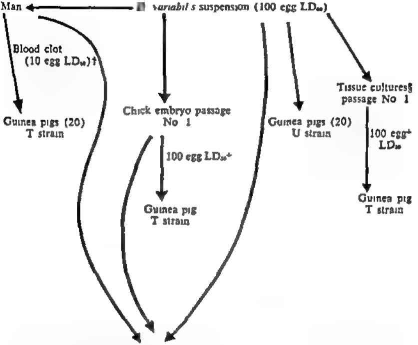
The avirulent phase of strain R was readily found in ticks in nature by Spencer and Parker [3] We have found the avirulent phase of the R S and T strains in ticks collected in the field

Studies we have carried out in Montana indicate that in the field the change from the avirulent to the virulent phase only comes about when the infected ticks feed However it is possible that in some warm areas the change from the avirulent to the virulent type might be brought about by the high temperatures since reactivation can occur by leaving the infected tick at 37 for 24 hours in the laboratory [3] It is also possible reactivation may occur when ticks infected with the avirulent phase do not feed on an animal but simply remain near the skin

Table II

THE EFFECT OF PASSING THE EASTERN U STRAIN IN HUMANS CHICK EMBRYO AND TISSUE CULTURE

Isolations of R rickettsii *from* D variabilis *and man*



* Titer of original tick suspension per 0 5 ml 0 5 ml of the suspension was inoculated intraperitoneally into 20 guinea pigs

† Titer of blood clot from patient per 0 5 ml of suspension 0 5 ml was inoculated intraperitoneally into 20 guinea pigs

‡ Dose inoculated intraperitoneally into 20 guinea pigs as determined by egg titration

§ Human testicular material was used

variabilis tick which had infected a human This tick was engorged with blood It may be seen that the strain isolated from the tick was the U strain but that isolated from the patient was of the T strain Therefore after one passage in man *the virulence of the strain was increased It should also be* noted that the virulence increased to the T strain after one egg passage This increase in virulence after one egg passage of the U strain is characteristic for the eastern part of the United States It is not clear whether the increase in virulence after one egg passage in man in the case of the eastern

Table 10

THE EFFECT OF ANIMAL PASSAGE BY MEANS OF SPLEEN TRANSFER AND ARTHROPODS ON THE VIRULENCE OF THE R TYPE OF *R rickettsii**

Animal	*Spleen transfer*	*Arthropod transfer*
Cotton tail rabbit	S and T types (8–11)	R (15)
Opossum	T types (6–10)	R (15)
Dog	T types (10–13)	R (15)
Columbian ground squirrel	R types (13)	R (15)
Chipmunk	R types (13)	R (15)
Field mouse (microtus)	R types (10)	R (15)

* All tests carried out by inoculating guinea pigs intraperitoneally with the 10 and 100 egg LD_{50} (which is equivalent to about 10 or 100 guinea pig ID_{50})

Figures in parentheses refer to number of passages

animal to animal by nymphs of *D andersoni* then there is no decrease in the virulence of the R strains In the spleen passage the material was titrated in chick embryos and inoculated into guinea pigs All passage material was compared to equivalent doses of rickettsiae from the virulent R strain before they were passed and thus all comparisons are on a quantitatively equal basis It should be pointed out that after a few passages in guinea pigs the strains which became lower in virulence after being passed in cotton tail rabbits dogs and opossums reverted to the virulent R strains The above experiments show that if we use the mode of transmission that operates in nature arthropods we do not change the characteristics of the strain This experiment also emphasizes the difference between the effects of artificial and natural transmission of an infectious agent

Experiments were also carried out to determine if larvae nymphs or adult *D andersoni* ticks infected with the virulent R strain and fed on guinea pigs or Columbian ground squirrels showing high CF and toxin neutralizing spotted fever antibodies would affect the virulence of the strain No effect was noted and indeed the rickettsiae multiplied in the ticks about as well as they did when fed on animals not containing any antibodies The only effect of antibody that has been found that would play a role in the maintenance of *R rickettsii* in nature is that animals containing complement fixing and toxin neutralizing antibodies to *R rickettsii* cannot infect uninfected ticks even though infected ticks are also feeding on the animal Such experiments have been carried out with Columbian ground squirrels and chipmunks Furthermore no effect on the virulence of either the R S or T strain was noted when *D andersoni* ticks infected with these strains were fed on a variety of animals with varying susceptibility to Rocky Mountain spotted fever

The only instance in which we have found a mechanism for modifying the virulence of a strain under conditions that exist in nature is the following case shown in Table 11 A strain of *R rickettsii* was isolated from a *D*

Table 12

THE EFFECT OF GROWING VIRULENT SPOTTED FEVER STRAINS IN *Haemaphysalis leporis palustris*

THREE VIRULENT STRAINS X A AND B ISOLATED FROM *D andersoni* WERE PASSED IN *Haemaphysalis leporis p lustris* TICKS FOR THREE COMPLETE LIFE CYCLES TWENTY PIGS WERE USED TO TEST EACH VIRULENT STRAIN

	100 LD_{50}		3 000 LD_{50}	
Material inject d	*Scrotal reaction*	*Fever*	*Scrotal reaction*	*Fever*
X	2+	2+	4+	4+
A	2+	+	4+	4+
B	2+	2+	4+	4+
X in H lp ticks	2+	2+	4+	4+
A in H lp ticks	2+	2+	4+	4+
B in H lp ticks	2+	+	4+	4+

Egg LD_{50} of tick suspension per 0 5 ml inoculated intraperitoneally into the guinea pig

Table 13

THE EFFECT OF GROWING *Haemaphysalis leporis palustris* STRAINS IN *D ande soni*

TWO *Haemaphysalis lepor s palustris* STRAINS WERE PASSED THROUGH 4 COMPLETE LIFE CYCLES IN *D andersoni* TWENTY PIGS WERE USED TO TEST EACH *Haemaphysalis leporis palustris* STRAIN

	100 LD_{50}		5 000 LD_{50}*	
Material injected	*S rotal reaction*	*Fever*	*Scr tal reaction*	*Fever*
H lp strain 1	1+	1+	+	2+
H lp strain 1 in *andersoni*	1+	1+	2+	+
H lp strain 2	1–	1+	1+	1+
H lp strain 2 in *andersoni*	1–	1+	1+	1+

* Egg LD_{50} of tick suspension per 0 5 ml inoculated intraperitoneally into guinea pigs.

D variabilis using ground squirrels and field mice to feed the larvae and nymphs and dogs to feed the adults since these conditions would closely approximate the conditions in nature no decrease in virulence was noted in the R strains

Summary

(1) Four main strains of *R rickettsii* can be di tinguished in nature in the United States

(2) The three strains showing virulence for the guinea pig have been found to exist in an avirulent phase in their arthropod vector in nature The main mechanism for increasing the virulence of the avirulent phase in ticks found in the field appears to be a blood meal though apparently the tem

low virulent strain or the increase in virulence in the case of the western rabbit tick strain after one egg passage is due to selection mutation a combination of these two phenomena or to adaptation The increase in virulence under these conditions is not understood in relation to the reactivation phenomenon since these low virulent strains were isolated from ticks which were full of blood and should have been in their most virulent form

It should be emphasized that when the original isolations of *R rickettsii* from arthropods collected in the field behave like a U strain in the guinea pig it is not possible to tell whether it actually is a U strain or is an avirulent phase of an R S or T strain since the avirulent phases of these three strains behave like a U strain It is only possible to say after attempts have been made to reactivate the organisms by one chick embryo passage and by one blood meal in a tick

The final series of experiments reported in this paper were carried out to see whether the arthropod vector influenced the virulence of *R rickettsii*

In all the experiments that will be described the particular tick species that was being studied were ground into a suspension in lots of about 3 to 4 ticks each at the appropriate arthropod passage and titrated in chick embryos 0 5 ml of the tick suspension was also inoculated intraperitoneally into 20 guinea pigs The reaction of these guinea pigs was compared to guinea pigs injected intraperitoneally with equivalent doses of the rickettsial strain before arthropod passage In all tests an original tick isolate was used in order to approximate as closely as possible the conditions that exist in the field Three hundred ticks were tested for each tick species studied

Only low virulent strains have been isolated from *Haemaphysalis leporis palustris* in nature Since the rabbit tick is a totally different specie than *D andersoni* and since it feeds only on rabbits and birds it could be that either the animal host or the tick might be responsible for the low virulence of the isolated strain Thus since we have studied the effect of rabbit passage on virulent R strains we were interested in studying the effect of arthropod passage of the virulent R strains in *Haemaphysalis leporis palustris* Three virulent strains were carried through three complete life cycles in *Haemaphysalis leporis palustris* using the cotton tail rabbit for all tick feedings From Table 12 it is apparent that there is not a decrease in virulence of the R strains under these conditions

It was thought of interest to see whether the low virulent rabbit tick S strains could be increased in virulence after passage through *D andersoni* since many virulent strains have been isolated from this tick After four complete life cycles in *D andersoni* in which either guinea pigs or ground squirrels were used for all tick feedings no increase in virulence was observed with the low virulent rabbit tick strains as shown in Table 13

Finally since *D variabilis* has yielded over 90% of the low virulent U or T strains in the field we have attempted to see whether this tick could decrease the virulence of the R strains After four complete life cycles in

6 Price W H "The epidemiology of Rocky Mountain spotted fever I The characterization of strain virulence of *Rickettsia rickettsii*" *Am J Hyg* 58 248–268 (1953)

7 Price W H "Interference phenomenon in animal infections with rickettsiae of Rocky Mountain spotted fever *Proc Soc Exper Biol & Med* 82 180–184 (1953) Price W H Unpublished experiments

8 Price W H "A quantitative analysis of the factors involved in the variations in virulence of rickettsiae" *Science* 118 49–52 (1953)

9 *Schlesinger R W Developmental stages of viruses" Ann Rev Microbiol* 7 83–112 (1953)

perature of 37 C associated with getting the blood meal is of the most importance in reactivating the organism

(3) Other experiments indicate that the virulence of the organism in its arthropod vector is not only controlled by the temperature but also by the molting hormone of the tick

(4) Areas containing high or low virulent strains of *R rickettsii* exist in nature the virulence of each area remaining relatively constant year after year

(5) Areas of a combination of high and low virulent strains also exist in nature In the two such areas studied the ratio of high to low virulent strains remained constant over the three year observation period

(6) Experiments carried out to approximate field conditions as nearly as possible have indicated that the interference phenomenon may play a role in the natural history of Rocky Mountain spotted fever by preventing mutant strains from arising The interference phenomenon may also help explain why some localities in nature have been found to have only one strain of *R rickettsii* over the three year observation period

(7) In a large series of experiments transovarial transmission occurred about 30 per cent of the time In these positive instances only a certain fraction of the eggs were found to have rickettsiae

(8) *D andersoni* infected with *R rickettsii* and fed on ground squirrels and chipmunks showing a high titer of complement fixing and toxin neutralizing antibodies failed to infect uninfected *D andersoni* fed on the same animals

(9) The fraction of the total ticks that are infected in nature may vary widely from year to year

(10) Attempts using animal and arthropod passage to change the virulence of any strain of *R rickettsii* by methods used in the natural mode of transmission have yielded negative results This of course does not mean that various species of animals or arthropods do not select strains of a certain virulence in nature but indicates that such changes do not occur readily

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2 Wolbach H B Studies on Rocky Mountain spotted fever *J Med Res* 41 1–197 (1919)

3 Spencer R R and R R Parker Studies on Rocky Mountain spotted fever *Hyg Lab Bull* 154 1–116 (1930)

4 Parker R R Maintenance of the virus of Rocky Mountain spotted fever in nature with particular reference to conditions in the Bitter Root Valley *Bull Montana State Board of Health* 26 33–40 (1923)

5 Topping N H "The epidemiology of Rocky Mountain spotted fever" *New York State J Med* 47 1585–1587 (1947)

here to follow the agent from its entry in a new host to its point of departure where we commence our discussion

Sites for Virus to Leave the Infected Host

In Table 1 have been tabulated a variety of sites for virus to leave the infected host

Table 1

SITES FOR VIRUS TO LEAVE THE INFECTED HOST

I Excretions or secretions from body orifices or superficial organs
- A Oro-nasal orifices
  - 1 Respiratory epithelium
  - 2 Pharyngeal surface
  - 3 Tongue and buccal ulcerations
  - 4 Salivary glands
- B Anal orifice
- C Urethral orifice
- D Vaginal orifice
- E Skin and mucous membrane
- F Eye
- G Breast orifice

II Covered sources not available at body surface or natural orifices
- A Blood
- B Ovary or uterine tube
- C Tumor

A very large number of viral agents leave the host through the oro-nasal cavities The immediate source of the virus is extremely varied and in some instances not well defined Several come directly from parasitized and damaged cells of the respiratory epithelium The pathology they produce there may greatly facilitate their excretion by such mechanisms as cough and coryza as in measles influenza and the common cold Others like poliomyelitis virus are present on the surface of the pharyngeal mucosa possibly by centrifugal spread from nerve fibers but with no recognized local pathology and are not usually aided in leaving the body by the presence of copious secretions coughing or sneezing Another group comes from ulcerative lesions on the tongue or other areas of mucous membrane in the mouth such as in foot and mouth disease vesicular stomatitis and probably in herpes and herpangina Still another group multiply in glandular tissue of one or more of the salivary glands some with and some without obvious pathology and are excreted into the mouth from the gland ducts These include mumps rabies and a variety of other less well studied mammalian viruses

Only a few years ago the number of viruses known to be excreted from the gastro-intestinal tract was very limited This is no longer the case and undoubtedly many more viruses will be found From the number now classed in the Coxsackie group it begins to appear that the variety of viruses in the gut might closely parallel the gram negative rods of the enteric group

17

Pathogenic Mechanisms of Virus Diseases

W McD Hammon

Graduate School of Public Health University of Pittsburgh Pittsburgh 13 Pennsylvania

We can well afford to marvel at the great variety of mechanisms which nature has provided to permit the survival of viruses Their completely obligate parasitism complicates their struggle for existence in passing from one host to another and requires an almost unlimited multiplicity of pathogenic mechanisms As discussed in previous talks of this series their requirements for nutrition their ability to penetrate certain cells and their resistance or susceptibility to the physical and chemical environment while not occupying an intracellular position necessitate this large variety of patterns which they as a group must employ to maintain themselves In this struggle for survival pathology is occasionally produced the factor which has led to their recognition Undoubtedly there are many other viruses probably those best adapted to survival which fail to produce pathology or do so infrequently thus they have not been recognized Fortunately under the title Pathogenic Mechanisms those which produce no pathology need not be included

No attempt will be made to cover the plant viruses First priority will be given to human pathogens but where better examples are available in the field of diseases of other animals these will be referred to Emphasis will be placed on mechanisms Examples will be restricted to one or a few for each mechanism For the sake of completeness we will include the *psittacosis lymphogranuloma* group of agents closely related to though considered by many not to be true viruses

We shall begin to trace the virus as it leaves the infected host then consider how it is transferred to the next host and finally how it gains admission to that host Pathogenesis has been and will be discussed by others so though equally important to complete the cycle no attempt will be made

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equine abortion and in lymphocytic choriomeningitis infection of mice there is some evidence that Newcastle virus of chickens enters the egg possibly from the oviduct or ovaries Dr Shope has told you of influenza infection of the lung worm within the chest of swine and how it leaves the host in the worm s ova—another unusual method of leaving the host s body Myxomatosis virus appears to be available from the skin-covered tumor tissue with access to biting insects These are adequate examples of the many sites and mechanisms by which a virus may leave the body of its host en route to another

Mechanisms for Virus Transfer to New Host

At this point in the cycle of infection another series of mechanisms may come into play In some instances transmission is extremely direct and rapid while in other instances it is highly indirect and the time interval may be long Some of these means are presented in Table 2

Table 2

MECHANISMS FOR VIRUS TRANSFER TO NEW HOST

I Direct
- A Droplets
- B Kissing nose contact by animals
- C Cannibalism
- D Suckling at breast
- E Skin and mucous membrane contact
- F Bite wound
- G Reproductive organs

II Indirect
- A Air borne particles
- B Contaminated objects water and food
- C Arthropods
  - 1 Mechanical
  - 2 Biological
- D Inoculation by contaminated needle surgical instrument or contaminated medication
- E Transfer of blood or blood product
- F Intermediate helminth

Droplets as such probably convey infection to any significant degree only from the oro-nasal portal of exit and when ejected directly in coughing sneezing and speaking This mechanism serves only at very close range and is probably of little comparative importance only for those viruses which cannot survive except for the briefest interval outside of susceptible cells or for those viruses which cannot withstand drying Poliomyelitis virus is one of those which cannot remain viable when dry Droplet carriage (moist medium) would thus appear to be more effective for this virus than air borne (dry) carriage whether the latter was originally from either throat or fecal source

Among other recognized human viruses excreted in the feces are poliomyelitis and infectious hepatitis The psittacosis agent also of importance to man is excreted from the cloaca of birds Russian spring summer encephalitis virus is excreted in the feces of its normal rodent hosts It has always been assumed that the viruses causing the common childhood diseases (measles chicken pox mumps etc) were not excreted in the feces but I know of no evidence in support of such an assumption Remarkable as it may seem the exact tissue sources or pathology associated with excretion of known viruses from the intestines are still unrecognized In this area there remains much to be elucidated

From the urinary tract common examples are lymphocytic choriomeningitis virus in mice and man canine hepatitis and distemper in dogs and several other species

From the vaginal orifice of women is excreted the agent of lymphogranuloma venereum

From the skin are shed the many pox viruses the virus causing warts and that of molluscum contagiosum and from the hoofs of cattle foot and mouth disease virus External skin pathology is present in each recognized instance Similarly from lesions of the mucous membrane the virus of herpes simplex venereal warts and others are spread also from areas of recognized pathology

From the eye examples are trachoma and epidemic keratoconjunctivitis

From the mammary gland is recognized the virus (milk factor) active in carcinoma of the breast in mice

We shall next consider a very large but varied group of those viruses not readily available at the surface of the body or excreted or secreted from body orifices and therefore requiring special methods to be set free for the infection to be spread From the blood are available yellow fever virus the arthropod borne encephalitis viruses homologous serum jaundice virus of man and a host of others Here again we encounter a large number of viruses present in this tissue in high titer but the original source of these viruses the cells in which they multiplied and the pathology produced if any remain to be determined In fact in many we do not know whether the viremia plays any essential part in pathogenesis or whether its sole function is that of serving as a source for an arthropod vector It has been pointed out recently and we shall probably hear about it subsequently that poliomyelitis of man may have a viremic stage and it is postulated that viremia is essential to its invasion of the nervous system I would like to point out that viremia has been adequately established and studied intensively in many of the other diseases in animals and man yet its role in pathogenesis is usually expressed with uncertainty

Among those infections with an occasional visceral mechanism of transfer it appears that small pox and measles may in some instances have intrauterine access to the fetus a new host This also occurs in infectious

cretions are still moist should be quite an effective mechanism of transfer In measles less complete experimental data on drying are available but epidemiologic observation suggests that clothing and hands of parents and doctors do not play an effective role as vehicles We conclude that the virus does not survive drying for more than a brief interval if at all Small pox and distemper virus on the other hand should survive well and prove infectious from dried secretions on contaminated articles or in food Moist fecal contamination of articles and food in all probability plays an important role in poliomyelitis spread in communities where there is a lack of good sanitary precautions in the care of fecal excretions

A fly cockroach or other invertebrate may also effect mechanical carriage from excreta on feet proboscis or more indirectly by passage through the intestinal tract in feces As far as can be determined no virus multiplication occurs under such conditions The importance of mechanical vectors is difficult to evaluate

Many viruses are carried indirectly by means of a biting arthropod from skin covered tissues This may be by simple mechanical means i e the soiled proboscis of a mosquito which has pierced a myxoma tumor in a rabbit More frequently this intermediate host has susceptible cells which serve to support virus growth and an extrinsic incubation period of several days at least is required before transmission can occur In no instance in the field of animal viruses to my knowledge is there any reliable information on the cells selected by the virus and pathology in the vector is unrecognized though well searched for in some arthropods An important consideration during this period of development in the vector is the environmental temperature under which the infected arthropod remains In the cold the vector though infected will fail to become infective while in high summer temperatures and those of the tropics the conditions for virus multiplication are ideal and within a week or less the vector becomes infective At intermediate temperatures incubation is prolonged and due to this epidemics are unlikely to occur The source of infection for the biting arthropod is usually blood

Mechanical carriage by a syringe needle a syringe full of blood or contaminated surgical instrument may replace the arthropod Homologous serum jaundice virus as far as has been determined is completely dependent upon such an artificial means of carriage A viremia of exceedingly long duration appears to be one factor making this possible and great stability of the organism in withdrawn whole blood serum plasma and certain chemically fractionated components also contributes significantly It would appear probable that mechanical transmission by biting insects must have played an important role before the days when transfusions blood counts and the use of blood products became commonplace in medical and surgical practice

A simple common mechanism with advantages to delicate viruses similar to those of droplet spread and frequently of far greater importance is direct contact (1) with lips *mouth or tongue of the infected host* (2) direct contact of the nose with genitalia and anus in canine hepatitis (3) skin contact in man for warts and molluscum contagiosum (4) venereal contact in lymphogranuloma venereum and venereal warts and (5) cannibalism or predation among animal species A similarly direct mechanism is suckling at the breast to acquire the milk factor in mouse carcinoma A bite is usually essential to carry rabies contaminated saliva effectively to *susceptible tissues*

A placental break may also serve as a direct mechanism of carriage of certain viruses to the fetus in utero The method of transmission from bird to egg or tick to its eggs remains to be determined

Air borne virus particles as droplet nuclei or on dust particles represent indirect or delayed transmission The minute dessicated particles may come originally from any of the sites of excretion previously discussed not just *from those of the respiratory tract In such diseases as measles and influenza* they probably most frequently come from the respiratory tract In these instances all epidemiologic evidence points to the necessity of a very brief time interval in the indirect exchange By contrast the air borne mechanism may serve more effectively for the highly resistant pox viruses which have been long in the dust after having fallen from the skin originally contained in a scab or from other sources The psittacosis agent also able to exist *for more than the briefest interval in the dried form frequently is* blown about in the air by beating of the bird s wings The agent is present in virulent form in the dried cloacal discharges settled on the perches and the bottom of the cage and readily becomes air borne

More indirect and effective for short or long periods of time depending on survival characteristics of the agent is the contamination of inanimate objects or fingers or of food or water It should be pointed out that in the contamination of food subsequent conditions are distinctly different than in many instances of bacterial contaminations and thus the mechanism is much less effective for there can be no multiplication of the virus Living susceptible cells are essential for any growth medium Contamination of *these many vehicles of infection may be gross or microscopic from excretions of nose or mouth from feces urine or secretions of the eyes or skin* It is believed that poliomyelitis measles chicken pox mumps infectious hepatitis distemper epidemic keratoconjunctivitis and a long list of others may be spread through such an indirect mechanism To evaluate the importance of the time factor for each agent one must know about physical and chemical conditions required for survival of the virus Laboratory evidence indicates inactivation of poliomyelitis after drying so microscopic dried fecal contamination would probably be harmless while gross mucous or salivary contamination of articles exchanged by children while the ex

contaminated fingers linen and contaminated eye medications and instruments

The skin serving as a barrier to most viruses is nevertheless readily penetrated by mechanisms necessarily adapted by some Skin contact or a superficial wound may serve for the wart virus a superficial wound may also serve for rabies virus in saliva However a tooth bite with salivary contamination is more frequently the effective mechanism in rabies transmission In most instances where skin penetration is required a biting arthropod or syringe needle injects the virus Surprisingly enough the specific cells which are first invaded after inoculation are still undetermined for most diseases In work with the arthropod borne viral encephalitides we found that subcutaneous inoculation was as effective or more effective than intravenous injection even though viremia is the only demonstrable sign of infection a few hours later

If time permitted we might consider all these mechanisms in other ways but with a few minutes to spare I would like to explore another mechanism which interests me particularly at the present

What role may immunity active or passive play in the transmission chain? In other words if the exposed host is immune what role if any may he play as a carrier? Two possibilities exist First an immune host may completely resist reinfection and fail even to get an immunologic boost from the exposure Or he may be reinfected and though remaining without clinical disease as a result of his previously acquired immunity still serve as a healthy carrier

We feel quite certain that an individual with immunity acquired actively resists reinfection with measles and after an appropriate incubation period does not serve as a healthy carrier The same appears to be true for chicken pox These conclusions have been drawn of necessity from epidemiologic analysis not by laboratory tests It would also appear that passive immunization for measl s of a nature adequate to prevent clinical disease also prevents infection the carrier state and the development of active immunity Thus we conclude that in measles immune individuals do not serve in a biologic role of healthy carriers in an infection chain However the evidence is quite strong to support the belief that passive prophylaxis in reasonable dosage in infectious hepatitis does not prevent infection the carrier state and the development of active immunity even though clinical disease is suppressed

In the field of the arthropod borne viruses there are some pertinent data on possible reinfection In yellow fever and most others these data indicate that viremia does not reoccur in an immune individual as a result of a bite by an infective vector Neither is a phenomenon parallel to that of Brill s disease in rickettsial infections recognized with viremia recurring in an immune host without recognized reexposure We have studied these phenomena with several of the arthropod borne encephalitis viruses in a num

Mechanism for Virus to Enter the New Host

Our final concern is that of the mechanism of entry into the new host (Table 3) In many instances this is obvious from the mechanism of trans fer but a variety again is encountered The natural body orifices play an important role

Table 3

SITES FOR VIRUS TO ENTER THE NEW HOST

I *Body orifices*
 A Nose
 B Mouth

II *Superficial organs*
 A Eye
 B Unbroken skin and mucous membrane

III *Subsurface tissues*
 A Wound
 B Arthropod bite
 C Artificial injection

The nose may be a receptor for air borne and droplet carried virus par ticles If susceptible cells are not present in the epithelial lining of the nose however the virus may well be trapped here and never produce infection Virus borne on dust particles stands small chance of passing this moist labyrinthine barrier while much smaller particles (droplet nuclei) are less likely to impinge and to fail to enter the pharynx bronchi or lungs where susceptible tissue may be present The mouth therefore may serve as a more satisfactory portal of entry for both air borne particles and droplets providing of course it is open during inspiration or at the appropriate moment to catch direct droplets Viruses entering the mouth in these man ners depending on their tropisms may find susceptible cells in the mouth the throat farther down the respiratory tree or farther down the gastro-intestinal tract Those virus particles which are not directed to the cells which they are capable of entering and which will support their growth remain ineffective and have failed in their search for a new host

By the mouth enter also the viruses from many contaminated vehicles including fingers toys water and food Here also is lip or tongue contact from kissing Here also enters the mouse milk factor and the pig s in fluenza virus within the earthworm and the lung worm larva This is a route shared in common by so called respiratory and gastro-intestinal viruses and in a number of instances we lack knowledge regarding the cells or organ where the penetration occurs For example in poliomyelitis one group believes the virus enters nerve fibers in the pharynx while others as sume that it emplants in susceptible cells at the level of the intestine and there initiates multiplication

The eye may also serve as a portal of entry for droplets for viruses from

apparently hold the view that immunity completely prevents reinfection for in a recent report they calculate inapparent infection rates in a child population by excluding all persons with detectable antibody from the population denominator

This problem has more than academic interest at the present! If actively acquired immunity prevents the carrier state following exposure to virus and if vaccination with inactive virus leading to antibodies of the same level also prevents subsequent infection we can reasonably expect possibly within the next decade almost complete interruption of the necessary mechanism to maintain natural inapparent immunization by poliomyelitis viruses At present this mechanism cares for most of the needs for immunity in the adult population At a price of a few young children developing clinical disease almost all older children and adults are immune probably for the rest of their lives If we should eliminate most child susceptibles from the population by vaccination and naturally acquired infection is thus greatly reduced periodic vaccination may be required throughout life Dangers of sensitization phenomena will be greatly increased and many may fail to continue immunization for many obvious reasons This could lead to occasional serious outbreaks among adults However continued vaccination of an artificially immunized child population throughout life will not be required if active immunity does *not* prevent the carrier state as a source of natural supplementary more permanent immunization As stated before we hope to have at least a partial answer to this question from a linear study of exposed immune individuals from the Philippine studies In the meanwhile there are a number of epidemiologists who believe that epidemiologic observations give reason to postulate that active immunity will not necessarily interfere with reinfection the carrier state and a boost to existing immunity At present I consider this to be a very important question

In conclusion it is readily apparent from this review of a few highlights of various pathogenic mechanisms of virus diseases that although much of a very useful nature is known about many of these diseases and their agents knowledge of all the mechanism involved in the total cycle is far from complete for most of them Some of these gaps in our knowledge may appear relatively unimportant at present but it is my opinion that much of very vital significance remains to be discovered and every part of the puzzle has great importance I hope that this review of some of the knowns and unknowns as seen by an epidemiologist will stimulate an interest and present a challenge to some working on closely related problems and that the challenge will lead to more answers

ber of laboratory mammalian and natural avian hosts and have never detected viremia (healthy carrier state) in any previously immune animal. Thus unless the mosquito possesses a mechanism superior to those ordinarily used in the laboratory for separating any possible active virus from an excess of antibody in the blood serum an immune host does not become a virus carrier in the group of arthropod borne viral diseases However xeno-isolation needs further investigation before this possibility is completely ruled out for certain hosts particularly the avian hosts in the encephalitis virus group These hosts might serve as an overwintering reservoir for the virus from which mosquitoes could liberate antibody bound virus in the spring

For sometime we have been interested in the possible carrier role of immune persons in the epidemiology of poliomyelitis Possible reinfection of immunes was the subject of a paper we presented several years ago Reinfection and the healthy carrier state were postulated on the basis of epidemiologic evidence principally on observations made among Guamanian natives We reasoned that in a small isolated island population with antibody rates approaching 100% in all over two years of age the virus would disappear (and it obviously had not) unless immune hosts participated in the infection chain The agents producing measles mumps and whooping cough could not survive in this same population group once they immunized a high proportion of persons Other available data also lent support to this concept of reinfection among immunes though artificial infection of laboratory animals did not More recently we have been attempting to determine the effect on infection of the extremely low antibody levels resulting from an injection of gamma globulin Naturally exposed gamma globulin injected children were studied using similar gelatin injected children as controls Those studied became exposed in families where cases were recognized These studies are still incomplete but indicate at present that infection the carrier state and active immunization do frequently occur during a period when passive protection is at a level usually adequate to prevent paralytic disease In fact the proportions of virus isolations in the two injected groups are identical at the present time

What happens to the actively immune person with a much higher antibody level from a previous type specific infection is now the subject of a large longitudinal study underway at an air force base in the Philippines After testing some portion of the 35 000 specimens being collected we hope to have a clear cut answer In feeding experiments with chimpanzees reported by other workers reinfection with homologous type of virus within a limited period of time after the primary infection usually does not occur In a recent report by Bodian on feeding large quantities of virus to two chimpanzees already possessing antibody from probable previous natural infection it appeared that active naturally acquired immunity prevented reinfection with a virus of the homologous type Melnick and Ledinko

apparently hold the view that immunity completely prevents reinfection for in a recent report they calculate inapparent infection rates in a child population by excluding all persons with detectable antibody from the population denominator

This problem has more than academic interest at the present! If actively acquired immunity prevents the carrier state following exposure to virus and if vaccination with inactive virus leading to antibodies of the same level also prevents subsequent infection we can reasonably expect possibly within the next decade almost complete interruption of the necessary mechanism to maintain natural inapparent immunization by poliomyelitis viruses At present this mechanism cares for most of the needs for immunity in the adult population At a price of a few young children developing clinical disease almost all older children and adults are immune probably for the rest of their lives If we should eliminate most child susceptibles from the population by vaccination and naturally acquired infection is thus greatly reduced periodic vaccination may be required throughout life Dangers of sensitization phenomena will be greatly increased and many may fail to continue immunization for many obvious reasons This could lead to occasional serious outbreaks among adults However continued vaccination of an artificially immunized child population throughout life will not be required if active immunity does *not* prevent the carrier state as a source of natural supplementary more permanent immunization As stated before we hope to have at least a partial answer to this question from a linear study of exposed immune individuals from the Philippine studies In the meanwhile there are a number of epidemiologists who believe that epidemiologic observations give reason to postulate that active immunity will not necessarily interfere with reinfection the carrier state and a boost to existing immunity At present I consider this to be a very important question

In conclusion it is readily apparent from this review of a few highlights of various pathogenic mechanisms of virus diseases that although much of a very useful nature is known about many of these diseases and their agents knowledge of all the mechanism involved in the total cycle is far from complete for most of them Some of these gaps in our knowledge may appear relatively unimportant at present but it is my opinion that much of very vital significance remains to be discovered and every part of the puzzle has great importance I hope that this review of some of the knowns and unknowns as seen by an epidemiologist will stimulate an interest and present a challenge to some working on closely related problems and that the challenge will lead to more answers

18

Pathogenesis of Variola

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Introduction

Smallpox may be regarded as the classical type of the exanthematous infections It has a fairly constant incubation period a well defined clinical picture and because of its relatively high mortality its morbid anatomy and histology have been extensively studied

On the basis of clinical and epidemiological observations and studies on *post mortem* material authorities such as Councilman [2] Ricketts and Byles[9] and Paschen[8] expressed views on the pathogenesis of smallpox which more recent work with improved facilities for investigation have not greatly modified

Because of the severity of the disease it does not lend itself to study by artificially induced infection in man as has been done in the milder infections of mumps measles and influenza Indeed in England intentional infection of man with variola virus has been illegal since the practice of variolation was abolished in 1840 although it seems that variolation was recently and perhaps still is practiced in some parts of China [10] However the causal virus of smallpox can now be readily isolated and handled in the laboratory and relatively simple tests are available for the assessment of antibody The experimental study of pox diseases of animals such as those of Fenner on mousepox and myxomatosis (if this latter be accepted as a pox disease) has also provided useful hints on the probable course of infection of the human pox disease The views to be presented take account of recent laboratory observations on smallpox material and on these experimental studies in animals There are however gaps in the picture which still have to be filled in by guess work

Clinical Course of the Disease

May I remind you of the clinical course of the disease Figure 1 shows a temperature chart from one of the cases in the Glasgow outbreak of 1950

Lesions appear as macules in the skin about the third day of illness and with the development of the eruption the temperature drops about the 4th or 5th day and the patient feels much better The extent of the secondary rise of temperature varies with the severity of the case In fatal cases who survive for ten to fourteen days as some do the secondary rise of temperature may be pronounced in milder cases the secondary temperature rise may be slight and in many cases of variola minor and variola major in vaccinated persons may be absent

The Incubation Period

Between the time when infection is acquired and the onset of illness a period of 10–14 usually 12 days elapses We have no knowledge of where the virus is multiplying during this period The virus probably enters the tissues through the mucosa of the respiratory tract and although it has been postulated that the virus multiplies in a primary focus in the respiratory mucosa there is no direct evidence of this The histological study of the

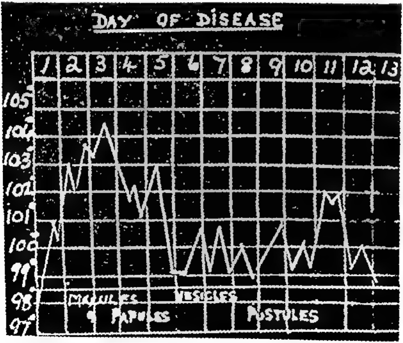

FIG 1 Temperature chart from a severe case of Smallpox

lungs in fulminating cases shows no such focus and the patients are not infectious during the incubation period Infected contacts have been allowed freedom of movement among their fellows during their incubation period without spreading the disease [5] So that if a primary focus is present in the lungs or upper respiratory passages virus is not shed from it in infective amounts to the environment It is possible as has been suggested by Fenner s experimental studies on mouse pox that the virus passes from the site of initial lodgement to lymph glands and after a short period of multiplication there enters the blood stream from which it is quickly removed by phagocytic cells in liver spleen bone marrow and other tissues After a further period of growth in these cells the virus is liberated again into the circulation at the end of the incubation period

Viraemia and Onset of Illness

Viraemia occurs in all cases at or just before the onset of illness We have made no examinations of blood for virus from patients during the incubation period to determine the presence or absence of virus in the peripheral circulation at this time but observations on congenitally acquired smallpox suggest that infection of the foetus *in utero* through the placental circulation occurs about the time of onset of illness in the mother and not before

Table I

VARIOLA—INTRAUTERINE INFECTION

Mother *Days from onset illness to delivery*	*Infant* *Day of life rash appeared*	*Onset of illness mother to rash in baby*	
6	7	13 days	Dixon 1948
3	9	12	
7	6	12–13 "	Marsden and Greenfield 1934
7	6	1 –13	
5	8	12–13	

Table 1 shows data on a few cases taken from the papers of Dixon and Marsden and Greenfield [7] These were cases in whom illness began in the mother before delivery and the rash appeared in the baby within ten days of birth so that infection was almost certainly acquired by the infant *in utero* through the placental circulation In the smallpox artificially acquired through the practice of variolation the interval between inoculation and the development of the rash was 9–12 days If we allow a similar interval in these cases of congenital smallpox because of the route of infection—an assumption perhaps not entirely justified—then it is apparent that the viraemia in the mother responsible for the infection of the baby *in utero* was about the time of onset of the mother s illness and not before

As might be expected the degree and duration of the viraemia varies with

the severity of the case We have made observations on this phase of infection by inoculating specimens of venous blood on the chorio-allantois of chick embryos The amount inoculated from any one case has varied from about 0 2 ml to 0 6 ml of blood distributed among several eggs The lesions which appear three days later on the chorio-allantois are quite typical

Figure 2 shows the combined results obtained in Liverpool and Dr Mc Callum s laboratory in London by this technique [3]

In patients who did not die of their disease virus was isolated only in four and in these on the first or second day of the disease In none of these were there more than 100 egg infective doses of virus in 1 0 ml of blood In some instances specimens were not inoculated on eggs until 12 to 24 hours after the bloods had been withdrawn If larger volumes had been cultivated without delay the proportion of positive isolations would have been higher

In cases which ultimately proved fatal it will be seen that virus was isolated from many more patients and that positive results were obtained later in the disease Moreover the amount of virus was greater and ranged up to 10 000 infective doses in 1 0 ml of blood In some of these patients antigen could be detected in the blood by complement fixation technique [3]

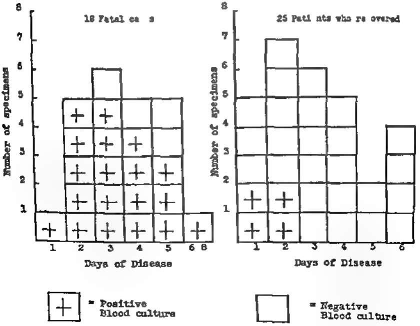

FIG 2 Viraemia in Smallpox Results of 52 blood specimens from 43 patients

Even in severe cases the amount of virus in the blood tends to diminish after the first day of illness In three patients from whom three successive daily samples were taken the amount of virus diminished from the first to the third specimen

Lesions in the Skin and Mucous Membranes

The viraemia at the onset of illness leads to infection in the first place of the capillary endothelium of the subepithelial vessels The earliest change to be seen histologically is the swelling of endothelial cells and a slight infiltration of mononuclear cells around these vessels In fulminating cases the destruction of vascular endothelium may result in the extensive haemorrhage seen in cases of purpura variolosa The macules which appear in the skin on the third or fourth day of illness may be due in part to infection of vascular endothelium or more likely may be the early stage of the vascular reaction to infection of the epithelial layers of the skin In any event by the time macules are present considerable growth of virus has occurred in the lower epithelial layers and scrapings of these macules show innumerable virus particles in stained smears At this time because of the impermeable nature of the upper layers of the skin virus is not shed to the surface and the skin lesions are not infectious in the average case This does not apply

FIG 3 Section of skin from a case of haemorrhagic Smallpox 3 days

however to the lesions in the mouth and pharynx When the skin rash is at the macular stage lesions in the mucous membrane of the mouth are breaking down and virus is present in the saliva We have not recovered virus from the mouth before the skin eruption appears at this time the majority of patients become infective The early skin lesion is illustrated by Figures 3 and 4

These show sections from a man who died after three days illness and showed numerous haemorrhages in the skin The typical focal rash was not detectable on clinical examination Histologically haemorrhage was apparent from small vessels in the cutis and in the epithelium small compact cytoplasmic inclusions are to be seen in cells obviously showing cytoplasmic degeneration In a neighbouring area inclusion material was more plentiful and filled the cytoplasm of ectodermal cells this is the appearance usually seen in sections from patients who die at later stages of illness The nature of such inclusion material is better shown in three day lesions on the chorio-allantois of infected chick embryos In these sections the granular eosinophilic inclusion material is almost certainly composed of masses of virus particles

By the time the skin eruption has reached the vesicular stage or possibly

FIG 4 High power magnification of Figure 3 Note cytoplasmic inclusions in lower epithelial cells

earlier, probably all cells to be destroyed by virus have already been infected The subsequent stages of pustulation and crusting are evidence of an inflammatory reaction to the necrosis of epithelial cells previously invaded by virus

Antibody Response

I mentioned earlier the fall of temperature and improvement in the clinical condition of the patient about the fourth or fifth day of illness This coincides with the development of antibody to the virus Indirect evidence of antibody formation at this time is provided by the results of vaccination of smallpox cases during the incubation period and the early days of illness Figure 5 shows the results of vaccination in many hundreds of patients recorded by Ricketts[9] in variola major and by Marsden[6] in variola minor There is a sharp drop in the number of successful vaccinations from the onset of illness

Antibody may in fact be demonstrated in the serum of patients by several techniques We have used the neutralization test the complement fixation test and antihaemagglutination technique in the examination of over 100 sera from smallpox patients The neutralization test was made by inoculating mixtures of sera and variola virus suspension on the chorio-allantois of groups of chick embryos The percentage reduction in the number of lesions as compared with control negative serum virus mixtures provides a sensitive

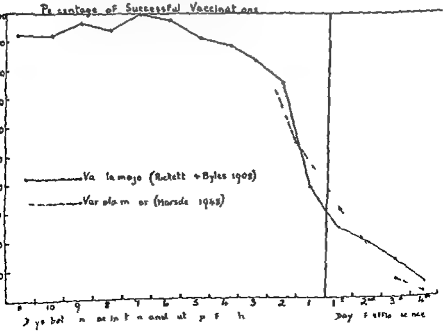

FIG 5

method for detecting minimal amounts of antibody Neutralizing antibody can be detected before antihaemagglutinin and this test appears to be a little more sensitive than complement fixation In smallpox cases occurring in previously vaccinated and in unvaccinated persons the antibody response in these two groups differs both in time and degree Figure 6 shows the results of neutralization tests for antibody in unvaccinated patients during the first fourteen days of the disease With one exception no antibody was detectable before the fifth day but after the fifth or sixth day most tests

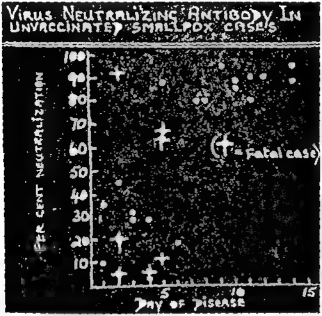

FIG 6

were positive (We have regarded 50% neutralization as our base line for the method showed a variation of 20–30% above and below the mean when counts of replicate mixtures of the same serum and dilutions virus were made on groups of eggs) The one exception was a woman of 44 who showed neutralizing antibody on the second day of illness She had a severe infection and died on the fourteenth day She had no record of having been vaccinated and no vaccination scar could be found when she was examined on admission to hospital

In patients who developed smallpox in spite of previous vaccination usually many years before antibody was present earlier in the disease and in greater amount (Fig 7) Antibody was detectable in most from the third day onwards The three patients who died were all men over the age of 50

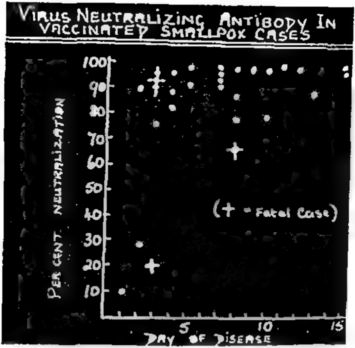
VIRUS NEUTRALIZING ANTIBODY IN VACCINATED SMALLPOX CASES
PER CENT. NEUTRALIZATION
100
90
80
70
60
50
40
30
20
10
(+ = Fatal Case)
5
10
15
DAY OF DISEASE

Fig 7

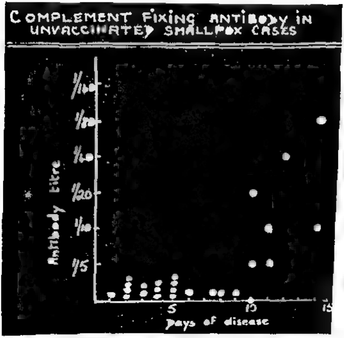
COMPLEMENT FIXING ANTIBODY IN UNVACCINATED SMALLPOX CASES
Antibody titre
1/5
1/10
1/20
1/40
5
10
15
Days of disease

Fig 8

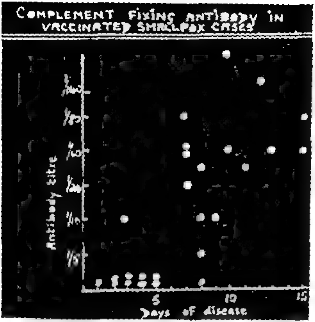
COMPLEMENT FIXING ANTIBODY IN VACCINATED SMALLPOX CASES
Antibody titre
5
10
15
Days of disease

FIG. 9

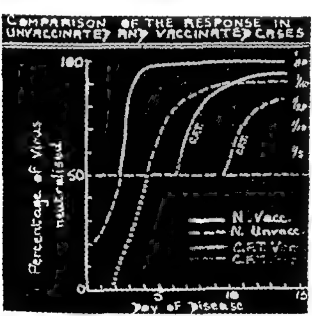
COMPARISON OF THE RESPONSE IN UNVACCINATED AND VACCINATED CASES
100
50
0
Percentage of Virus neutralised
C.F.T.
C.F.T.
N. Vacc.
N. Unvacc.
5
10
15
Day of Disease

FIG. 10

who had not been vaccinated since childhood Figures 8 and 9 show the findings on the same sera by complement fixation technique

Antibody was not detected by this technique before the tenth day in unvaccinated patients but was present by the 7th day in those who had been previously vaccinated These results are combined in diagrammatic form in Figure 10 which indicates the more rapid and greater response in the vaccinated persons who developed smallpox

Antibody Formation in Relation to Progress of Disease

It would appear then that even in unvaccinated cases antibody can be detected in the blood serum about the fifth day of illness at a time when the clinical condition of many patients improves In the previously vaccinated in whom the antibody response to the secondary stimulus of infection is more rapid and greater improvement in the patient s clinical condition may continue with little or no secondary fever the evolution of the lesions may be quicker than usual

In the recent smallpox outbreak in Yorkshire four cases were diagnosed as *variola sine eruptione* Each had been successfully vaccinated some years previously and later showed a good antibody response to the smallpox infection In such cases presumably virus growth proceeds to the viraemic stage when antibody is present in the blood by neutralizing the virus antibody prevents infection of skin epithelium so that no skin eruption occurs

In the moderately severe and severe cases the progression of the lesions through the pustular stage may be associated with return of fever and deterioration in the patient s condition and in some of these death may occur towards the end of the second week In most of these cases however the spread of virus has probably been halted earlier at about the fifth or sixth day of illness and further infection of cells has not taken place The virus already inside cells protected from the action of antibody will continue to grow and produce cell necrosis The progress of the lesions through the stage of pustulation and crusting is presumably the inflammatory response to the products of cell destruction By the time antibody appears infection of epithelial and other cells may have been so widespread that the consequent cell necrosis has serious effects even to the extent of leading to a fatal issue—in spite of the presence of antibody Secondary bacterial infection may be of little importance We have rarely isolated bacteria by blood culture in smallpox patients and the use of antibiotics has not greatly reduced the mortality from variola major

In other diseases a similar state of affairs may exist in that the appearance of antibody does not coincide with clinical recovery In experimental mumps Henle *et al* [4] have shown that antibody may be present at the onset of clinical symptoms and in poliomyelitis the appearance of antibody in the blood may not prevent the development of severe paralysis

Two patients whose sera contained antibody on the second and third

days of disease (Figs 6 and 7) died of their infection One unvaccinated person already referred to died on the fourteenth day the other a man of 61 vaccinated in infancy died on the fourth day of a severe purpuric form of the disease One must suppose that in these patients there had been extreme proliferation of virus with widespread infection of cells before or at the time of onset of illness and before the appearance of antibody The severity of the illness will on this view be determined largely by the progress of infection up to the first or second day of illness

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DISCUSSION

Ecology and Pathogenesis of Virus and Rickettsial Infections

DR SABIN (Moderator) I think that everyone here shares my own feeling of admiration in seeing so heroically conceived and beautifully executed a series of tests as have been shown on these two lantern slides I m sure that we will be able to get more of the story that Dr Kunkel has to tell during discussion We must proceed now and ask Dr Winston Price to speak on the subject The Influence of Host and Environment on Rickett siae

DR PAUL Dr Shope s paper illustrates the fact that the modern microbiologist does not spend all of his time in the laboratory but is allowed to put on his boots or overshoes and go into the field in order to round out his observations on infectious disease

In reference to Dr Audy s paper one might call attention to its applica tion not only to *cleared* forests but to areas where the forest and bush have recently *returned* This happened in Korea during the recent war where in 1951–53 behind the stabilized front there was an area perhaps fifty miles

in depth which previously had been intensively cultivated for centuries This was suddenly abandoned in 1951 and as a result it completely changed the ecology of the area providing a new type of cover for bugs insects and rodents which probably had its impact on the local prevalence of certain microbial diseases

DR SALK I would like to comment on Dr Shope s very excellent presentation this morning Dr Paul just indicated that it would be quite in order to put boots on and find out more about the ecologic aspects of disease in nature There are some agents of disease that are pathogenic only for man and reside in man as the exclusive reservoir In the case of human influenza one wonders where the virus is in inter epidemic periods over a period of several years we attempted to search for this needle in a haystack First we tried to reduce the size of the haystack by looking for the agent where we thought that it might possibly be present This we did by examining sputum from patients who had pneumonia that was presumed to be and in fact was bacterial pneumonia We were able to find evidence for the existence of the influenza virus in a number of such patients even in an inter epidemic period This has led us to make the suggestion that the provoking factor for human influenza as Dr Shope has so admirably demonstrated for swine influenza may well be a bacterial infection It may well be that the virus exists in some persons in a non pathogenic form and survives in this masked form The agent under proper stimulation may then emerge in a pathogenic form to cause disease to the patient himself and then may spread to the rest of the community

DR SABIN Thank you I think one of the purposes of discussion is to have stimulating speculation or suggestions on the part of those who are not working in the immediate field I would like to take advantage of the presence of people working in phage to get their comments on the masked form of virus to which Dr Shope referred We naturally think of the similar interesting phenomena in bacteria e g prophage and lysogenesis Has any one here among the bacteriophage people been stimulated to make some interesting comments and suggestions that may help in our concepts of the nature and form of the masked virus? Do we have some comments on that? Or have all the bacteriophage people retired to see Detroit today Dr Hershey

DR HERSHEY I can t say very much In fact I think it is too soon to draw analogies between masking as seen in animal and bacterial viruses All we phage people know about our own material is that there are several kinds of masked bacteriophage The most important of these are the vegetative phage found (or rather not found) during the eclipse period in the growth of virulent phages the prophage that must be assumed to account for the

perpetuation of virus in lysogenic bacteria and the host induced modification of phages This last class is the newest one but different examples have already been described by Luria Weigle Bertani Krueger and Anderson What one finds is that when a virus passes through a new host it may emerge as a fully infective particle but showing a markedly narrower or broader host range than it did after passage through the previous host This effect is non hereditary all the particles liberated from a given host are characteristic of that virus growing in that host

Perhaps one more instance of masking should be mentioned here (added in proof) which I suspect may be closely related to the host induced modifications Here the result is the same but the conditioning factor is not the host itself but the presence of a second virus in the host The effect of the mixed infection is to cause nonheritable modifications in the host range of both viruses Since the modifications are *toward each other* the phenomenon is called phenotypic mixing and one sees a vague clue to possible mechanisms Streisinger has recently shown that both the host range and tail serotype of T2 and T4 are subject to phenotypic mixing but that several other characters by which these two viruses differ are not

DR WOOLLEY Perhaps since Dr Hershey is the only phagologist to respond I thought I would tell you something of the way a non phagologist is thinking about these things In the experiments we did with T2 bacteriophage some four or five years ago it was found that if you take E coli B and grow it in a medium containing citrus pectin and infect that medium with T2 phage the bacterial organism grows very well and is not lysed In fact it grows as well as if there were no phage there Similarly the phage grows as well as if there were no pectin Here one has a sort of happy community of bacterium (E coli B) and phage T2 The thing which preserves stability is the pectin Several other polysaccharides have been shown to function similarly in this virus host community

The way I would argue from such a phenomenon of co-existence of virus and bacterium is the following If normally E coli were producing some polysaccharide like citrus pectin it would get along perfectly well with phage T2 If now through some low temperature or other insult to the coli its mechanisms for the production of adequate amounts of this polysaccharide were interfered with then it would be destroyed by the phage I could not help but think of this when Dr Shope mentioned his experiments with pigs on the roof in the cold weather Perhaps the pigs just failed to make some polysaccharide and the virus took over

DR PUCK I would like to add to Dr Hershey s list additional parallels in bacteriophage action to the phenomenon of masked virus T4 and T6 phages do not attach to their host cells unless the medium has the specific molecule L-tryptophan present In the presence of this particular metabolite

the phages are fully invasive in its absence they are completely without effect on the host Another effect which has been described by Dr Sagik working with Dr Luria is a masking of T2 phage by some molecular components as yet unidentified which appear to originate from the host cell material This inactivating factor can cause the titer of an initial lysate to be depressed by a factor from 10 to 100 times of what it would be when this material is removed The masking material can be removed by exposure to distilled water or by treatment with a negatively charged surface like a cationic exchanger These procedures raise the titer by a hundred fold Finally the phenomenon of T1 sensitization in which the phage can be reversibly inactivated by the presence of an abnormal concentration of certain inorganic cations may also at least formally be considered a reactivable masking of the virus

DR SABIN Apparently there are a number of things that might be done with cercaria and lung worms to try and bring out the masked virus Now this does not conclude the discussion of this morning s papers because there must be many interesting questions and comments directed to Dr Kunkel and Dr Price We shall have another discussion period after the last paper and this morning s contributors will have an opportunity to come back We shall cut the intermission period by several minutes

DR HORSFALL It has been a long time since we here have had an opportunity to hear from Dr Downie I have been distressed to discover that this morning he made no mention of the very important findings he has unearthed recently regarding variola virus I speak of his evidence that variants can be derived from this agent I wonder if you would permit him to talk for a minute or two on this important subject

DR DOWNIE I am very sorry to disappoint Dr Horsfall The variants we have been studying recently are variants of cow pox virus—strains derived from the natural disease of cattle—and not variants of variola virus though naturally we have been looking at that with a view to finding the same kind of variant So far we can t say we ve had very much success

DR SABIN Would you while you have the microphone tell us what you think alastrim is?

DR DOWNIE Although the diseases variola major and minor are clinically and epidemiologically distinct alastrim virus so far as our laboratory observations go is indistinguishable from the virus of variola major We have examined it in chick embryos and in animals We have done extensive serological tests including cross neutralization by various techniques and we find no differences between the two So much so that when an outbreak of

smallpox occurs in England we cannot tell by laboratory examination whether it is alastrim or variola major That can only be determined I think by the clinical picture and the subsequent course of the epidemic

DR SALK I would be interested in hearing something about the variants of vaccinia if Dr Downie would be willing to expand on that Dr Horsfall had asked him only about variola

DR SABIN Will all the speakers please hold their questions and replies We will try to go around systematically Any more questions now please? We will try to have all the comments and the discussion later I would like to get the questions now first

DR SAMUEL SASLAW (Ohio State University) I would like to direct this question at Dr Hammon If we are going to consider primary atypical pneumonia as a virus disease has this disease disappeared temporarily or is it just a local observation that we have made during the past three years it has just been extremely rare that we find it—can t demonstrate it to medical students—does the virus burn itself out or is there a temporarily immune population that will eventually become susceptible or is this just local observation? I would like to hear what s happening country wide

DR J W CZEKALOWSKI (Univ of Leeds England) I would like to ask Professor Downie about his view on the nature of reaction of hyperimmunized individuals after revaccination Some of our experiments carried out together with Dr C W Dixon (Dept of Preventive Med University of Leeds) suggest that this reaction might be of an immune nature

DR CHRISTINE E RICE (Animal Diseases Research Institute Department of Agriculture Hull Que) I would like to ask Dr Downie if he has used the indirect complement fixation test in titrating the antibody in these patients I ask that because we have been doing some work on the detection of antibody in foot and mouth disease in convalescent cattle We find that five days after the appearance of lesions the inhibitory activity of this antibody has been demonstrated by indirect complement fixation test In the occasional specimen we get antibody appearing which cannot be detected by the direct test until about three weeks The majority of these convalescent bovine serums never show direct complement fixation activity but in a group of about 34 animals in a naturally infected herd we found 30 developed strong inhibitory activity I know Dr Downie has used the indirect test in studying the activity of the variants

DR GINSBERG I would like to ask Dr Hammon whether he has any evidence on the resistance to second attack of measles of in patients who have had modified measles that is measles modified by gamma globulin

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DR HAMMON I have no answer to that either I haven t been currently observing it

DR SABIN Is there anybody here—Dr Loosli—would you comment on it please?

DR CLAYTON G LOOSLI (University of Chicago) We too are seeing less atypical pneumonia on our infectious disease service We saw several cases however during the respiratory disease season last winter We haven t encountered any so far this fall

DR HORSFALL I think there is very little doubt that atypical pneumonia has gone through a cycle rising in incidence from a low level in about 1936 to a really high level particularly in the armed forces about 1940 Since the war the incidence appears on a nation wide scale to have decreased strikingly and we hear from California Chicago Baltimore Boston and New York City that few cases are now being seen

DR SABIN How about an interpretation Do you have an interpretation of this phenomenon?

DR HORSFALL No none at all But I think probably it is more apparent than real and would wonder whether many patients were not treated at home as in general the disease is not severe and many would consider that it didn t require hospitalization The data we obtain is largely from hospitals

DR SABIN Any contrary interpretation that has a bearing on the problem of ecology?

DR SASLAW When I first posed this question at some of our medical staff meetings I inquired pretty thoroughly among all our internists in town too and they hadn t seen them at home either So we are still looking

DR SABIN Since we still do not have a specific test to diagnose infection with the virus of primary atypical pneumonia I think we probably cannot proceed much further Dr Audy do you have any comments or replies?

DR AUDY I should like to comment on Dr Paul s remarks earlier this morning in connection with the gradual restoration or near restoration of the original circumstances following the regrowth of cleared areas We have experimental evidence *to support his statements but this is not the place to* present it From what I know of Dr Paul s own writings I would suggest that he is warning us about the danger that confronts every epidemiologist

DR SABIN Thank you— Any other questions? I would like to direct a question to Dr Kunkel He pointed with great emphasis to the fact that the aster yellows virus cannot be transmitted *manually* except by grafting Then he went on to tell us about the way the insect transmitted the disease I think it would perhaps help us in our thinking if he could tell us what the insect does to the leaf when it feeds on it i e in a mechanical way? Why do you have to graft the virus when the insect doesn t do it? I would also like to direct a question to Dr Price Dr Price described most interesting observations on the occurrence of so-called avirulent forms in rickettsiae in nature and stated that one egg passage or 37° C for 72 hours could convert the avirulent to the virulent The question is are there any data to suggest that in the natural host these rickettsiae exist as a mixed microbial population and that what happens in the one egg passage or at 37° C in the original host is an overgrowth of one of the components? Or is it an actual transformation of the pre existing avirulent ones?

DR C H KEMPE (University of California Medical School) Dr Downie points out that the virus is present massively in viremia in fatal cases and only rarely in non fatal cases Since the difference between the two is presumably one of quantity and since we know that the virus is largely present in the white blood cells would it not be more intelligent in the future perhaps for all of us to try to isolate the virus from non fatal cases by using a volume of buffy coat 0 1 ml of buffy coat rather than 0 1 ml of whole blood in order to increase our chance since there is so little virus present in a non fatal case

DR HERBERT A WENNER (University of Kansas) I would like to ask Dr Kunkel another question in regard to the yellows aster virus From his charts I got the impression that perhaps the survival rate in the leafhopper was shorter after feeding for 14 days and I am a little curious to know whether this virus has an effect on the insect vector

DR SABIN One more question—anyone? The question period is concluded and with your permission I would like to ask each of the speakers to reply in the order of their appearance on the program if possible within about one minute so we can have five minutes left for interesting speculations and comments We will start with Dr Shope

DR SHOPE I am sorry I don t know the answer to the question that was directed by Dr Saslaw I believe to me but I think that Dr Hammon could answer it It concerned the incidence of atypical pneumonia

DR SABIN Dr Hammon would you answer the question about the incidence of atypical pneumonia?

DR KUNKEL One might think so but that is not the case I would like to point out that about a third of the well known plant virus diseases have not and apparently can not be transmitted mechanically except by grafting This is not an unusual phenomenon among the plant viruses

DR PRICE In regard to the question of how we prepared our antigen—the rickettsiae were harvested from yolk sacs purified by the centrifugation adsorption and the albumin procedure of Bovarnick and Miller and then killed by the addition of formalin

Dr Sabin asked whether the reactivation phenomenon could be due to selection This is of course an extremely important point We feel from the following data that reactivation is not due to selection

One lot of ticks was collected from one female and raised to nymphs They were infected as nymphs with a virulent strain of *R rickettsii* When they molted to adults they were put in the ice box If two adult *D andersoni* are left in the ice box for a few months and made into a suspension and titered they may show 1 egg LD_{50} per 0 5 ml 0 5 ml gives rise to no symptoms in guinea pigs and about 80% of the animals are immune to challenge with 10 ID_{50} of the virulent strain If two other ticks are taken from the ice box and left for 12 hours at 37 C they still may titer only 1 egg LD_{50} per 0 5 ml but 0 5 ml of this suspension when injected into guinea pigs results in typical virulent spotted fever If one assumes that reactivation is due to a few virulent organisms being present with many avirulent ones and the virulent ones grow during the reactivation phenomenon then the above two ticks must have contained at least 1 virulent rickettsiae If now one takes a larger number of ticks from the ice box and prepares a suspension so that it contains 300 egg LD_{50} per 0 5 ml there should be at least 300 virulent organisms present Since at a maximum you only need 100 organisms to cause virulent spotted fever 0 5 ml of this suspension should cause a virulent spotted fever reaction in guinea pigs Actually at the most the guinea pigs will show very slight fever for a few days I might add that if only 5 egg LD_{50} of the virulent reactivated rickettsiae which is equivalent to about 5 ID_{50} for the guinea pig is added to the suspension containing 300 egg LD_{50} of the avirulent phase the guinea pigs come down with virulent spotted fever Observations like these together with the fact that it is possible to obtain reactivation where no increase in rickettsiae can be demonstrated although under these conditions an increase of about three fold could be detected if it had occurred makes us feel that the evidence at the present time best fits the hypothesis that the strains of *R rickettsii* we have worked with can undergo a change to an avirulent phase for the guinea pigs and under suitable conditions may then become virulent again

I would also like to make one further point about the multiplication of the avirulent phase of *R rickettsii* prepared from refrigerated ticks Al

and especially every field worker that of seeing things only in a cross section Even when one works on a field problem for many years one is still investigating only a cross section It is essential to think backwards and forwards as well as sideways and to look upon the whole set up as a series of space time events The title of this symposium is salutary because it uses the word Dynamics our investigations too often give us static pictures and if we are not careful these give us static conceptions

DR KUNKEL In answer to the first question as to what the insect does it simply feeds It introduces its proboscis into the tissues until it finds the phloem of a fibro vascular bundle somewhere in a leaf There it feeds on the juices in the phloem which it regurgitates that s about all we know about it The insects are greedy feeders they feed almost continuously during the daylight hours—not at all at night and not very actively late in the afternoon or early morning In regard to whether or not there is an affinity between virus and insect we know very little We have been able to see no marked effect on the insect and the fact that those insects that feed for a long time on diseased tissues on the whole live a little shorter life than those that feed for a short time can be explained in other ways Diseased plants have bacteria and fungi on them and an insect that feeds on such plants for a long time gets some of these organisms or products from them at least In the diseased plant some of the cells collapse and food materials set free are fed on by bacteria and fungi so it is very difficult to judge from the length of life of the insect I think whether there is any ill effect from multiplication of the virus in the insect

DR SABIN While you still have the microphone Dr Kunkel may I ask you another question in view of your answer? Since the insect apparently does nothing more than introduce the virus as by needle or by injection are there any data to indicate that an extract of infected insects as well as extract of leaves cannot infect another plant by some procedure which would simulate the injection of the insect Why the grafting? Is the virus in some different form in the insect?

DR KUNKEL We doubt that it is in any different form but apparently it is in a much higher concentration in the juices of the insect than it is in the juices of the plant It is possible to pass this virus from insect to insect by needle inoculation It has been passed with great difficulty from plant to insect by needle inoculation but never from plant to plant or from insect to plant by needle inoculation or in any other way that has been devised and much effort has been spent on this

DR SABIN Concentration of virus would not explain it because if you could make an extract of a sufficiently large number of insects you ought to be able to transmit it by needle inoculation of the plant

talk about that All I can say is that there appears in our cowpox strains on the chorio allantois a white variant which appears with quite a high frequency The white variant isn t as virulent for the chick embryo as the normal haemorrhagic strain and it has a much slower growth rate consequently it never replaces—at least it hasn t under these conditions replaced —the more virulent parent strain

DR HAMMON My reply can be very brief Dr Ginsberg asked I believe whether I had any personal experience as to whether modified measles cases were immune on subsequent exposure and I suppose he means those that have been modified by gamma globulin I have no personal observations on that and I don t think Dr Ginsberg needs me to review the findings of others

though all attempts to demonstrate multiplication of these rickettsiae were negative as reported in this paper there is some evidence that these rickettsiae may multiply to a slight extent If one compares the complement fixation titers and toxin neutralizing titers of guinea pigs injected with equivalent doses of the avirulent phase and killed rickettsiae of the virulent phase the inoculation of the avirulent phase always results in higher antibody responses Whether this means that the ether and formalin which is used to prepare the killed vaccines partially inactivates some of the rickettsial proteins concerned in the formation of antibodies or whether it indicates multiplication of the avirulent phase is under study

DR DOWNIE I will try not to take too long sir Dr Kempe s question with regard to virus and blood cells We did in fact examine a good many of these bloods by separating cells and serum in other instances we lysed the whole specimen and tested that In the first day or two there was little or no difference between the distribution of virus in serum and in the cells but in one patient who had marked antibody on the third and died next day there was quite a lot of virus in the blood but it was all present in the cellular fraction according to our results But of course it was difficult to be sure that antibody was not masking virus in the serum Then Dr Rice s question We haven t been doing indirect complement fixation tests in examination of these human sera We used that technique a great deal in the study of fowl sera because with these we couldn t do direct complement fixation tests our experience with fowl sera comparing neutralizing titers and titers by indirect complement fixation test did not suggest to us that *the indirect complement fixation test would be more sensitive than the direct* complement fixation test on sera with which the direct test was possible Then Dr Czekalowski s question about the rapid disappearance of virus from the vaccination site in revaccinated people we have made no observations on this We do however revaccinate our staff every year or every two years we have frequently titrated antibodies in our sera by neutralization tests on the chorio allantois We have found marked rise in antibody even though the take on revaccination may be slight with a very tiny lesion vesicle just visible with a hand lens on the second or third day Then Dr Bell s question—about antibody and susceptibility to smallpox I m afraid we made no direct observations of this We have examined a great many sera of vaccinated people at different times after vaccination and as one might expect the titres in individuals vary quite markedly Some show antibodies 40 years after primary vaccination as determined by the neutralization technique but not by complement fixation other people in a few years—ten or fifteen years—seem to have lost the neutralizing antibody We made no direct observations on people who were going to get smallpox because we don t know who these are

The variant of cowpox virus—I m afraid it would take a long time to

Part III

Mechanisms of Immunity in Virus and Rickettsial Infections

Moderator

John Paul

Yale University School of Medicine
New Haven, Connecticut

19

Mechanisms of Convalescent Immunity and How It May Be Simulated*

Jonas E Salk
School of Medicine University of Pittsburgh Pittsburgh Pennsylvania

In considering the question of convalescent immunity and how it may be simulated it is clearly apparent that different mechanisms apply to the different infectious diseases including those caused by viruses It is the purpose of this presentation to consider first the mechanisms of convalescent immunity from the epidemiological viewpoint and then later to touch upon the cellular and humoral phenomena in the individual

Epidemiologic Patterns of Disease

The foundation for the present theme derives from an examination of the epidemiologic patterns of different diseases In the first 4 figures these are illustrated schematically and show the fluctuations in the indices of disease and also in the indices of the state of resistance over intervals of time for 4 different epidemiologic patterns These are patterns of (1) a recurrent epidemic disease (2) an endemic disease with regular periodic eruptions in younger age groups (3) a low grade endemic disease that has within recent times become a low grade epidemic disease and (4) a disease of low grade endemicity that does not cause epidemics

In these figures the index of occurrence of disease is in terms of the number or proportion of the population affected as manifested by overt cases Since we are interested not only in the *disease* itself and the reasons for its existence but even more in the state of *resistance* as these fluctuate in the course of time the activity of the disease is presented schematically

Studies on poliomyelitis referred to were aided by grants from The National Foundation for Infantile Paralysis

Studies on influenza were conducted under the auspices of the Commission on Influenza the Armed Forces Epidemiological Board and supported by The Office of The Surgeon General Department of the Army

and (2) why does not the entire population develop immunity to a disease that recurs so frequently and (3) why do epidemics occur We shall return to these questions again later

Another epidemiologic pattern is illustrated in Figure 2 The example in mind when this chart was prepared was paralytic poliomyelitis In the United States for example and in the Scandinavian Countries and in many others the transition of poliomyelitis from a low grade endemic disease to a low grade epidemic disease has occurred relatively recently at some time in the past the state of resistance of the population was uniformly high but in recent years this has been altered and epidemics now occur Again there are the interesting questions (1) as to where is the virus between epidemics and (2) the reason for epidemics as well as (3) the reason for the state of resistance

Another pattern (Figure 3) is suggested by the way in which measles manifests itself as an endemic disease and its tendency to recur quite regularly in large communities in sharp eruptions chiefly affecting the younger age groups Again manifestations of disease are indicated as the photographic positive and resistance by the "photographic negative"

Finally in Figure 4 there is the pattern of a disease of low-grade endemicity one that does not cause epidemics There are numerous examples of diseases of this kind such as influenza C Coxsackie infections

A LOW GRADE ENDEMIC DISEASE THAT HAS BECOME A LOW GRADE EPIDEMIC DISEASE

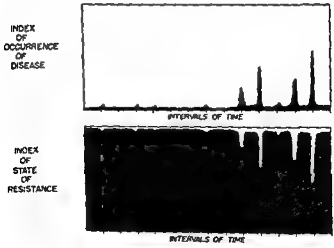


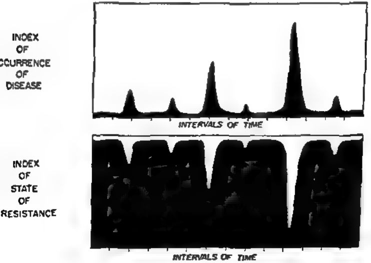
FIG 2

as the photographic positive and resistance as the photographic negative It must be mentioned that the state of resistance of a population as indicated by the proportion of persons who are not ill at any particular time includes those who are (a) immune, (b) refractory (c) not exposed (d) infected sub-clinically The principal group with which we are concerned is that which is immune in the immunologic sense Since it is difficult to determine for some diseases at least what fraction of the resistant population is resistant as a result of an earlier infection and since this is an essential question with which we will deal later it is important to mention in passing that this component will be segregated from the rest later in this presentation

To begin this discussion let us consider Figure 1 which illustrates a recurrent epidemic disease and the illustration is intended to point up the *features of influenza A If the intervals of time are taken as one year it* would appear that epidemics that differ in intensity tend to recur at more or less regular intervals This has been well documented in many reports The questions that come to mind when one examines both the upper and the lower half of the figure are (1) where is the agent between epidemic periods

EPIDEMIOLOGIC PATTERNS OF DISEASE AND OF IMMUNITY IN A HYPOTHETICAL POPULATION

A RECURRENT EPIDEMIC DISEASE



* INCLUDES THOSE WHO ARE
(a) IMMUNE
(b) REFRACTORY
(c) NOT EXPOSED
(d) INFECTED SUBCLINICALLY

This is like INFLUENZA A

FIG 1

A DISEASE THAT IS OF LOW GRADE ENDEMICITY AND DOES NOT CAUSE EPIDEMICS

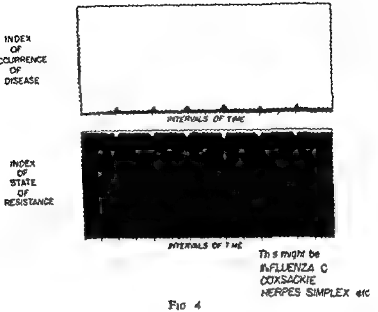


Fig 4

of its immunologic instability smallpox is a disease that has been eradicated from wide areas of the earth

Host parasite Relationships

Having considered the 4 patterns that have been presented let us now look at the viruses that cause these diseases The first question that might be asked is related to the residence of the virus or the form in which it exists in the inter-epidemic periods or even in endemic periods Without entering into a discussion concerning the nature of viruses and the theoretically different forms of their existence i e , incomplete forms or partially degraded forms let us use a general term to indicate a quiescent form by drawing upon the analogy with certain of the bacterial diseases and in this epidemiologic consideration use the term spore like pha e to characterize the quiescent state in the life cycle of the virus If we assume that such a state does exist and if we make a further speculation that viruses may be divided into 2 categories—namely those that are able to survive in a spore like phase either in (a) an immune host or (b) only in a non immune host we may summarize the types of epidemiologic patterns for agents that can behave in each way as in Figure 5

ENDEMIC DISEASE WITH REGULAR PERIODIC ERUPTIONS IN YOUNGER AGE GROUPS

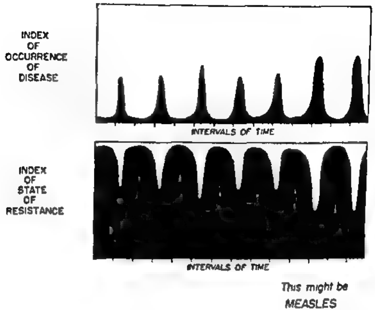


FIG 3

and many others This might well represent the pattern of the activity of agents that are not identified with an obvious disease this may be the way in which a disease manifests itself that is caused by the agents that are said to be in search of a disease

In reflecting upon the differences in the epidemiologic patterns of different diseases and of their associated immunity it would seem that these patterns may depend upon the nature of the host parasite relationship and more specifically upon *whether or not the agent can exist for longer or shorter periods either in an immune host or only in a non immune host* This concept is illustrated in Figure 5 where it may be seen that an immune host is defined as one who possesses serum antibody and a non immune host as one who does not possess demonstrable serum antibody Concerning viruses that have man as the only reservoir a corollary of the foregoing hypothesis is that the capacity to survive in an immune host indicates that the virus has solved the problem of survival Viruses that cannot exist in an immune host may either disappear from an immune population or if not have survived because they have acquired some other means for so doing—such as the capacity for immunologic variability An illustrative example of a virus that can survive in the presence of antibody is the virus of herpes simplex Influenza is an example of a virus that is able to survive because

early in life under the protective influence of maternal antibody and causes virtually no clinical disease but nevertheless as indicated by immunologic studies a constantly high rate of infection exists Thus it would seem that the poliomyelitis virus must be able to survive in an immune host or else the virus should have become extinct by virtue of the high proportion of immunes Poliomyelitis can also manifest itself in another way as indicated by the third example of a disease that can survive *in an immune* host that is as a cause of low grade epidemics The virus may behave in this way if the population infection rate is low thereby reducing the possibility of infection early in life under the protective influence of maternal antibody (when it is present) thus leaving the potential susceptible to acquire his first response to infection when he might develop concomitant paralysis

The other illustration of a virus that can survive in an immune host is that of the virus of herpes simplex which may be regarded as the cause of a low grade endemic disease This virus is constantly present in the population as manifested by the frequency of herpetic lesions especially in certain individuals The circumstances suggest that the virus exists in a latent form in the same host for long periods of time and that transformation from the spore like or latent phase to the active form is the result of any of a variety of influences the mechanisms of which are not clearly understood It would appear that viruses that are believed to survive in an *immune host* by and large constitute the group that cause the *low grade endemic* or *low grade epidemic problems* whereas viruses that are postulated to survive only in a *non immune host* are responsible for the *more prominent epidemic diseases*

The two examples selected of epidemic diseases are (1) measles with regular periodicity and (2) influenza with an irregular periodicity As for measles it is difficult to decide whether or not this virus is a pure example of an agent that can survive in a *non immune host only* or whether it may perhaps belong in the category of agents that can survive in an *immune host* But for the sake of the present discussion it is considered in the category of those that survive only in non immunes As for influenza this virus is included for reasons that will be elaborated upon shortly The other example of a virus that is postulated to survive only in a non immune host is that of smallpox It is theoretically possible for the virus of smallpox to become extinct within a population as a result of its own activity and would then require that the agent be re introduced from without On the other hand self-extinction or eradication seems not to occur either for poliomyelitis or herpes where the immune mechanism appears not to have eliminated these viruses from the populations in which they are active

In anticipating the later part of this discussion the conclusion that might be drawn thus far is that viruses that can survive only in a non immune host might conceivably become extinct or be eradicated by a world wide

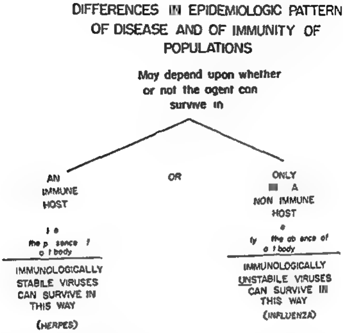


FIG 5

For viruses that can survive in an immune host it is possible that no clinical disease will ever be evident or at least at such a low rate as to appear to be non existent But in actual fact a high rate of infection does occur particularly in the early years of life The example for this may be poliomyelitis which in communities having a high rate of infection occurs

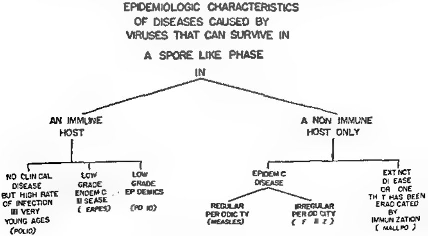


FIG 6

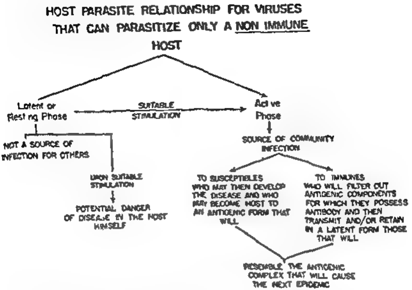


Fig 8

The host parasite relationship for viruses that can parasitize only a non immune host is illustrated in Figure 8 Viruses of this kind are postu lated to exist in a latent or resting phase during which time they are not a source of infection for others Upon suitable stimulation these agents do constitute a potential danger for the host and this is in contrast to the absence of risk to the parasitized host who possesses antibody as illustrated in Figure 7

In illustrating the sequence of events after the transformation of the latent or resting phase into an active phase by suitable stimulation where upon there is a source of infection for the community I have in mind the influenza virus This agent may be transmitted to susceptibles who are infected briefly during which time they develop the disease and then sub sequently a few may continue for longer or shorter periods of time as hosts to an antigenic form possessing the antigenic components that may be the cause of the *next* epidemic The mechanism that is postulated is that anti body to the predominant antigenic component of the infecting strain will neutralize the antigenic constituents for which an adequate antigenic stimu lus was induced by the infectious process but will allow certain variants to remain that may have been in the minority and that possess the capacity to exist in a latent or resting phase until some later time when under suitable stimulation they may then emerge as a cause of disease In a similar man ner during the period of activity of the virus while it is causing wide spread infection in the community it may be transmitted to persons who have some

immunization program whereas viruses that are capable of surviving in an immune host will in spite of immunization continue to survive and all that might be expected of immunization is that it may be possible to control those manifestations of infection that are disturbing—such as the paralytic sequel of infection with the poliomyelitis virus

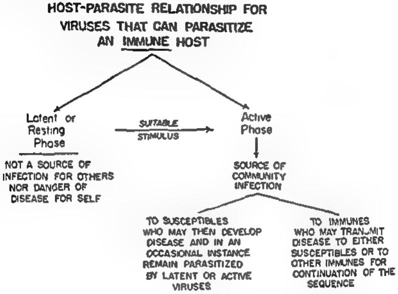


Fig 7

Let us examine further (Figure 7) the nature of the host parasite relationship for viruses that are able to parasitize an immune host During the period when the virus exists in a latent or resting phase it is presumed to be non transmissible and without danger to the host himself That the latent or resting phase virus is not transmissible is mere speculation but that the latent or resting phase virus is not of danger for the host is due to the fact that the host already possesses immunity to disease although he is obviously not immune to infection Suitable stimulation (to be considered later) will convert the latent or resting agent to the active form In the active form it may then serve as a source of infection for the community Under these circumstances the agent may be transmitted to susceptibles who may then develop the disease and in an occasional instance such persons may remain parasitized by either latent or active virus for longer or shorter periods of time and thereby maintain the cycle Alternatively the active virus which is the source of community infection may also be transmitted to immunes who themselves are resistant but who may develop a more or less transient parasitism and may transmit the disease either to new susceptibles or to other immunes for a continuation of this sequence

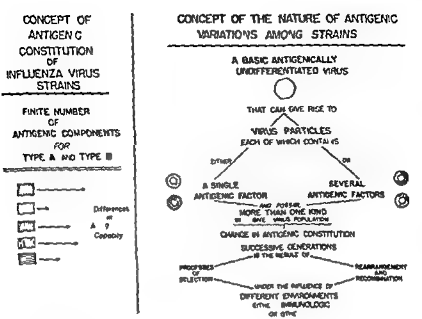

CONCEPT OF ANTIGENIC CONSTITUTION OF INFLUENZA VIRUS STRAINS
FINITE NUMBER OF ANTIGENIC COMPONENTS FOR TYPE A AND TYPE B
Differences
Capacity
CONCEPT OF THE NATURE OF ANTIGENIC VARIATIONS AMONG STRAINS
A BASIC ANTIGENICALLY UNDIFFERENTIATED VIRUS
THAT CAN GIVE RISE TO
VIRUS PARTICLES
EACH OF WHICH CONTAINS
A SINGLE ANTIGENIC FACTOR
SEVERAL ANTIGENIC FACTORS
MORE THAN ONE KIND
CHANGE IN ANTIGENIC CONSTITUTION
SUCCESSIVE GENERATIONS
SELECTION
RECOMBINATION
DIFFERENT ENVIRONMENTS

Fig 9

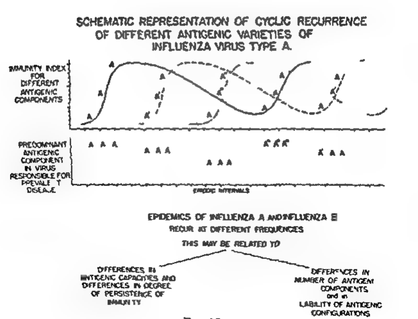

SCHEMATIC REPRESENTATION OF CYCLIC RECURRENCE OF DIFFERENT ANTIGENIC VARIETIES OF INFLUENZA VIRUS TYPE A.
IMMUNITY INDEX FOR DIFFERENT ANTIGENIC COMPONENTS
PREDOMINANT ANTIGENIC COMPONENT IN VIRUS RESPONSIBLE FOR
A A A
A A A
A A A
A A A
EPIDEMICS OF INFLUENZA A AND INFLUENZA B RECUR AT DIFFERENT FREQUENCIES
THIS MAY BE RELATED TO
DIFFERENCES IN ANTIGENIC CAPACITIES AND DIFFERENCES IN DEGREE OF PERSISTENCE OF IMMUNITY
COMPONENTS
LABILITY OF ANTIGENIC CONFIGURATIONS

Fig 10

degree of immunity and in whom the antigenic components for which they possess antibody will be neutralized Such individuals will then transmit or retain in a latent form a virus population that may be the precursor of the antigenic complex that will cause the *next* epidemic

This hypothesis may explain, perhaps the seemingly rapid spread of in *fluenza virus infections throughout wide areas of the world within so short* a period of time as to make one wonder how it is possible for an infectious disease to begin in one locality and spread so quickly and so widely The suggestion here is that the appearance of antigenically similar forms of the influenza virus in different parts of the world almost simultaneously represents the operation of the exact same phenomenon at the termination of the epidemics that affect widely separated populations at the same time or at different times chronologically but at similar times in relation to the cyclic recurrence of the disease The common denominators throughout are the occurrence of an epidemic at some previous time and then the operation simultaneously of similar factors that are responsible for conversion of the latent or resting phase into the active phase in corresponding geographic or climatic regions This does not mean of course that the emergence of an epidemic in one part of the world may not serve as a focus of infection for spread to other parts of the world but is intended merely to attempt to explain the almost simultaneous occurrence or emergence of similar antigenic forms in more than one focus at a time It would serve to explain too *the major transformation in the predominant immunologic form of* the virus of influenza A such as occurred in 1946–1947

To recapitulate it is interesting to note that agents that can successfully survive in an immune host—that is in the presence of specific antibody—are ones that are immunologically stabile whereas viruses that are not able to survive in an immune host must have some mechanism other than the capacity to exist in a latent form in order to survive The unique feature of the human influenza viruses which appear able to survive by virtue of their immunologic instability is the reason why influenza still continues as a major problem among the uncontrolled diseases whereas an *immunologically* stabile virus such as variola can become extinct or can be easily controlled by immunization

Virus Factors

To complete the picture of the way in which the influenza viruses behave there is illustrated in Figure 9 a concept of the nature of this virus The two essential features are (1) that a finite number of antigenic components exist and (2) that these may interchange as the major or dominating constituent under a variety of influences Figure 10 *illustrates the manner in* which the different antigenic components of a single immunologic type may exchange places first in the forefront and then by virtue of the increased immunity index resulting from the activity of that component would tend to

HYPOTHETICAL REPRESENTATION OF SEASONAL EMERGENCE OF PARALYTOGENIC VIRUS FROM IMMUNE CHILDREN OR ADULTS PARASITIZED BY A NON INFECTIOUS FORM OF THE POLIOMYELITIS VIRUS

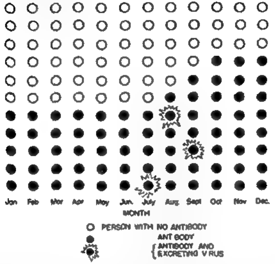


Fig 12

The emergence in certain seasons of a paralytogenic virus from children or adults who may be parasitized by a non infectious form of the poliomyelitis virus is described in Figure 12 In this hypothetical representation persons with no antibody are illustrated by the open circles the solid symbols represent persons who have acquired antibody as a result of a previous natural infection The irregular halo around the black symbols indicates excretion of paralytogenic virus Thus during the winter and early spring months in the upper regions of the temperate zone when paralytic poliomyelitis is not occurring in a major way the thought is that the agent may exist in a quiescent form in a few of the persons who were infected previously and who have continued to be parasitized just as are those persons who harbor the herpetic virus It is to be remembered that not all persons who were once infected with the herpes virus always develop lesions (at which time they can serve as a source of infection to others) Similarly the concept is that not all persons previously infected with the poliomyelitis virus will continue to serve as a reservoir for infection of others The minority who constitute this reservoir would be sufficient for the seasonal influences to act upon them and in the late summer or fall for them to serve as a source of infection for others who may then acquire a silent infection or a paralyzing

be suppressed allowing another component to emerge The first may re emerge at a later time when the immune state of the population will allow the earlier antigenic form to re emerge

Having illustrated the way in which influenza virus activity may wax and wane under external influences it is desired to illustrate hypothetically the way in which a virus that is capable of existing in a quiescent or latent form in an immune host might behave First let us consider the factors or conditions that may influence conversion of a latent to an active virus these are illustrated in Figure 11 Here it may be seen that seasonal factors play a role such as the tendency for swine and human influenza to occur in the winter time for poliomyelitis to occur in the summer time and for sun and wind that are in some instances associated with season to influence the emergence of the herpetic lesion Then there are hormonal factors such as those associated with pregnancy that have an influence upon whether or not poliomyelitis will or will not cause the paralyzing disease Then there are age and constitutional factors that influence susceptibility to poliomyelitis and the severity of the disease resulting therefrom There is the example of bacterial infection which can influence the conversion of the latent to the active virus i e the appearance of herpes simplex accompanying meningococcic meningitis or pneumococcal pneumonia or typhoid fever and evidence has been uncovered to suggest that a bacterial infection of the lung and possibly of the upper portions of the respiratory tract may perhaps cause the influenza virus which may be present in the latent form to emerge in an active form The latter is the converse of what is usually believed to occur—namely that influenza virus infections precipitate bacterial infections The concept here is that both may occur For completeness the possibility is cited but there is no substantial evidence as yet that some other virus infections may establish the pre-conditions that may influence the emergence of another virus that exists in a latent form

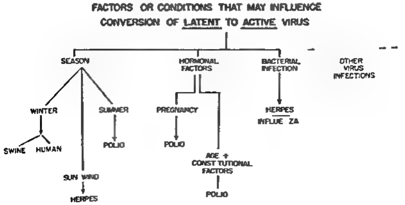


Fig 11

in life and most often will develop active immunity under the protective in fluence of the maternally-derived passive immunity Thus it would appear that under natural circumstances where a high immunity index exists poliomyelitis does not eliminate itself by creating a highly immune popula tion on the contrary it tends to perpetuate itself much more readily under such conditions

In the upper right hand portion of Figure 13 is illustrated schematically the situation that exists in populations in which paralytic poliomyelitis has become a low grade epidemic disease This appears to be the result of the separation of the host and the parasite and perhaps is due to the improved hygienic conditions that keep the virus from infecting not only the new members of the population but the older ones as well Nevertheless under these circumstances there exists at all times a much higher proportion of susceptibles immunologically speaking and therefore when contact is made with the poliomyelitis virus there is a greater opportunity for the occurrence of the paralytic form of the disease

These two diagrammatic representations have implications in regard to the question as to how poliomyelitis might possibly be controlled by im munologic means If one were to consider the use of a living virus prepara tion to be fed to individuals in a population in which a high proportion of children young adults and adults do not possess antibody then one would be increasing artificially the chances for contracting infection if it is assumed that the virus that is fed would spread from person to person If the agent used in this way is certain to be fixed as a non paralytic agent there would be no danger and this could solve the problem of artificial immunization However a question that will have to be answered is whether or not the path whereby either under natural or under laboratory circumstances relatively avirulent agents develop is a , one way or a two-way street

In the lower half of Figure 13 is illustrated the proportion of immunes with advancing age for a disease such as influenza A that tends to occur in epidemic form every two years with sporadic cases in between epidemics It is clear from this that in due course all or almost all individuals become infected and within a relatively short time this agent which is unable to parasitize in the presence of specific antibody has a reduced opportunity to spread On the other hand another antigenic variety of the same immu nologic type but one that has not been responsible for epidemics for many years will find a fertile field at all age levels The reason for this is based upon limited opportunity for immunization against the new variety which may have existed all along as a minor component of the previously prevalent virus this opportunity would have been had *only by individuals in the older* age groups who may have experienced one or more infections with the viruses that possessed this minor component

Thus it would seem that the concentration of convalescent immunes would influence the opportunity for manifestation of poliomyelitis and in

INFLUENCE OF CONCENTRATION OF CONVALESCENT IMMUNES* UPON THE EPIDEMIOLOGIC CHARACTERISTICS OF CERTAIN VIRUS DISEASES

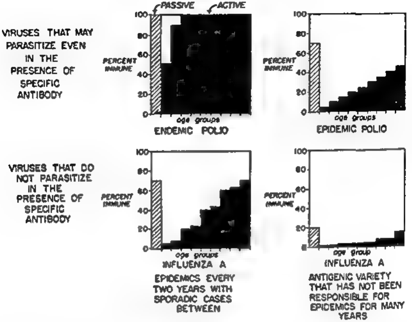


Fig 13

infection and in either instance serve to enlarge the reservoir that maintains the infection in the community The next illustration (Figure 13) shows the influence that the concentration of convalescent immunes has upon the epidemiologic characteristics of poliomyelitis if the mechanism that has just been described is operative

Influence of Concentration of Convalescent Immunes upon the Epidemiologic Characteristics of Certain Virus Diseases

Figure 13 shows schematically the immunologic status of individuals at different ages for a virus such as poliomyelitis that may parasitize even in the presence of specific antibody In populations in which poliomyelitis is endemic—that is to say a population in which the paralyzing disease occurs quite infrequently it has been reported by many observers that the immunity that exists in the adult appears to be associated with the presence of serum antibody Under these circumstances antibody can be passively transferred to the new born uniformly If a high rate of infection exists and the hygienic conditions are such that the host and parasite exist in intimate relationship then a high proportion of the young will become infected early

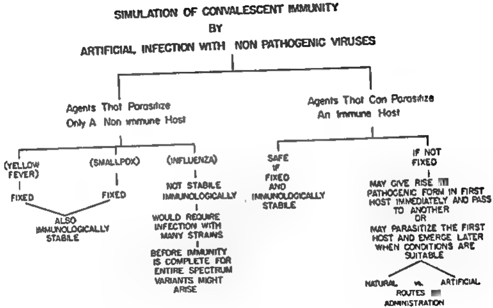

SIMULATION OF CONVALESCENT IMMUNITY
BY
ARTIFICIAL INFECTION WITH NON PATHOGENIC VIRUSES
Agents That Parasitize Only A Non immune Host
Agents That Can Parasitize An Immune Host
(YELLOW FEVER)
(SMALLPOX)
(INFLUENZA)
FIXED
FIXED
ALSO IMMUNOLOGICALLY STABLE
NOT STABLE IMMUNOLOGICALLY
WOULD REQUIRE INFECTION WITH MANY STRAINS
BEFORE IMMUNITY IS COMPLETE FOR ENTIRE SPECTRUM VARIANTS MIGHT ARISE
SAFE IF FIXED AND IMMUNOLOGICALLY STABLE
IF NOT FIXED
MAY GIVE RISE
PATHOGENIC FORM IN FIRST HOST IMMEDIATELY AND PASS TO ANOTHER
OR
MAY PARASITIZE THE FIRST HOST AND EMERGE LATER WHEN CONDITIONS ARE SUITABLE
NATURAL vs. ARTIFICIAL
ROUTES
ADMINISTRATION

FIG 15

fluenza in different ways in one case a high concentration of convalescent immunes would help to serve to maintain the agent in the population and the other would help to suppress it and force the emergence of an antigenic relative

Methods for Simulating the Convalescent Immune State

Having discussed the ways in which convalescent immunity influences the behavior of certain of the virus diseases at least from the epidemiologic viewpoint let us now consider how convalescent immunity may influence the occurrence of infection or disease in the individual This is summarized in Figure 14 where it may be seen that the presence of antibody resulting from

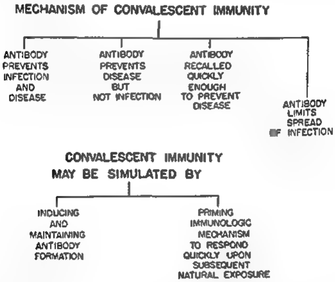


FIG 14

the convalescent immune state may prevent both infection and disease Antibody on the other hand may prevent disease but may not prevent the recurrence of infection The convalescent immune state may allow antibody to be recalled quickly enough to prevent the manifestations of disease for the second time and in some instances the antibody which is not able to prevent the establishment of infection may limit the spread of infection

If we consider that the convalescent immune state is mediated by humoral factors (i e antibody) and that the convalescent immune state may be simulated as illustrated in Figure 14 by (1) inducing and maintaining antibody formation or (2) by priming the immunologic mechanisms to respond quickly upon subsequent natural exposure then these can be achieved in one of two ways as illustrated in Figures 15 and 16

Figure 15 indicates how convalescent immunity may be simulated by artificial infection with non pathogenic viruses In keeping with the classification

Another way of simulating convalescent immunity is by inducing active immunity with a non infectious virus preparation For the application of this approach due consideration must be given to all factors both qualitative and quantitative that are important for inducing development and persistence of antibody formation It is theoretically possible to exploit the factors listed in Figure 16 for application to poliomyelitis in a way that has been found successful for the control of certain of the bacterial diseases particularly those due to the effect of exo toxins and also certain of the viral and rickettsial diseases that have been brought under partial or complete control by this means

The general principles of immunization with non infectious agents require that there be (1) a sufficient antigenic mass (2) a suitable method for destroying the infectivity or toxicity of the antigen (3) the availability of the antigen in a relatively pure form so as to avoid the interfering effect of extraneous antigens and it is necessary to employ (4) the booster phenomenon and/or (5) immunologic adjuvants either for adequate primary immunization or for long term effects It is desired to illustrate how this approach is being used in studies directed toward the development of methods for preventing influenza and poliomyelitis The approach to the solution of both of these diseases is similar in some respects however because of the special problem posed by each and because of the probable existence of a different mechanism for the long term maintenance of immunity in each it is necessary to illustrate them separately

Immunization against Influenza

Because of the (1) immunologic complexity of the influenza viruses and (2) the need for erecting a solid barrier against all immunologic varieties that have occurred in the past and (3) the need for adding those that may occur in the future (assuming that a finite number of antigenic varieties exist) a means has to be developed that will permit the inclusion in a single inoculum of a sufficient antigenic mass for each component without becoming involved in too concentrated a virus preparation that might be toxic or without reducing the antigenic mass to levels that would be inadequate A further requirement for immunization against influenza is the production of antibody to a level that would be adequate to overflow into the superficial secretions of the respiratory tract to permit the prevention of invasion of the susceptible respiratory epithelium The need for this exists because the infecting agent in this disease comes into contact with the susceptible cell without being transported through the blood stream where antibody is present Another special feature of influenza is the short incubation period which would preclude re infection acting as a booster stimulus after basic immunization which for some diseases is all that is required to fortify an immune effect that requires antibody for its mediation Therefore it becomes necessary to employ techniques that would raise antibody titers to

SIMULATION OF CONVALESCENT IMMUNITY
BY
INDUCING ACTIVE IMMUNITY WITH KILLED VIRUS

Due Consideration Must Be Given To All Factors
Both Qualitative and Quantitative That Are
Important For Inducing Development And
Persistence of Antibody Formation

SUFFICIENCY OF ANTIGENIC MASS	SUITABLE METHOD OF KILLING	PURITY OF ANTIGEN	BOOSTER PHENOMENON	IMMUNOLOGIC ADJUVANTS

FIG 16

developed earlier we will consider first agents that can parasitize only the non immune host The use of non pathogenic viruses for the control of yellow fever and for the control of smallpox has been possible because the non pathogenic variants of the natural disease producing agents appear to be fixed from the viewpoint of pathogenicity and these agents also appear to be immunologically stabile thus the multi potentiality for the emergence of different immunologic forms as in the case of the influenza viruses appear not to apply As for influenza which is not stabile immunologically it would require that infection be induced with many strains and there is the possibility because of the nature of the organism that before immunity is complete for the entire spectrum variants might arise which would be difficult to control except in strictly isolated communities

For the virus that can parasitize an immune host such an agent could be considered safe if it were fixed in terms of the loss of its disease producing powers and if it were immunologically stabile If the agent is not fixed in the pathogenic sense there is the possibility that it may give rise to a pathogenic form in the first host immediately and then pass to another or it may parasitize the first host without producing disease and emerge later in a pathogenic form when conditions are suitable Even though assurance is not had that the non pathogenic form is permanently fixed it is possible that administration of the virus by injection rather than by feeding which might be done for poliomyelitis might not result in excretion Under such circumstances it would be necessary merely to bring forth evidence to indicate that there is no danger to the host administration of the virus in a way that would avoid excretion and infection of others would eliminate any concern about the redevelopment of a pathogenic form upon human passage This of course is entirely hypothetical and is presented merely to illustrate all possibilities whereby convalescent immunity may be simulated by artificial infection with a non pathogenic virus

GEOMETRIC MEAN ANTIBODY TITERS AT INTERVALS UP TO TWO YEARS AFTER VACCINATION WITH INFLUENZA VIRUS VACCINE IN SALINE OR EMULSIFIED WITH MINERAL OIL

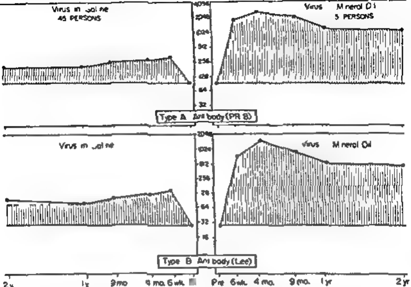


FIG 18

to high levels and then persist for reasonably long periods of time As for the duration of effect the degree of persistence of antibody over a 2 year period is illustrated in Figure 18 The subjects involved in this study are to be observed for a number of years

Among the questions that still need to be resolved fully in the application of this method of immunization are those concerned with the local cellular response accompanying the injection of a mineral oil emulsion and questions concerned with sensitization It appears that neither will be of clinical significance but only by further study can this be established with certainty Inoculations of such vaccine have been given to more than 30 000 subjects and studies are being continued with further refinements of emulsifying agents and of mineral oils and of the aqueous component of such vaccines

Immunization against Poliomyelitis

In considering the matter of immunization against poliomyelitis there are two major differences between this disease and influenza One is the likelihood that for the most part this virus may pass through the blood stream before gaining access to the central nervous system therefore low levels of antibody might be effective in preventing the paralytic consequences

sufficiently high levels and for it *to be maintained* above a critical threshold for long periods of time With these prerequisites various means have been explored and the most efficient and effective means that has been found for inducing antibody formation is through the use of immunologic adjuvants of the water in-oil type of Freund

The problem here is to develop a *clinically acceptable* combination of reagents that are included in the adjuvant mixture Toward this end studies have been in progress for several years and it now appears that the goal is being approached To illustrate the way in which the efficiency of the antibody inducing effect has been enhanced data are presented in Figure 17 to show the relationship between antibody level induced and the quantity of virus incorporated in an aqueous vaccine as compared with vaccine made with the mineral oil emulsion It is clearly evident from these data that by a proper selection of dose of each antigenic component and proper combination of those that are adequate as far as antigenic capacity is concerned as well as antigenic coverage it should be possible to prepare in a single inoculum a sufficiently potent antigen that will cause antibody to be raised

ANTIBODY RESPONSE TO DIMINISHING QUANTITIES OF INFLUENZA VIRUS VACCINE EMULSIFIED WITH MINERAL OIL OR IN SALINE

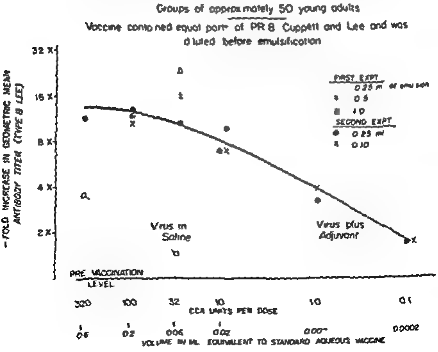


FIG 17

of this infection Another consideration is the probability that the incubation period for poliomyelitis is longer that that for influenza thereby allowing the possibility that infection may permit the booster effect to operate thus augmenting whatever antibody may be present in the serum from a previous immunologic experience and to do so before the central nervous system is invaded This possibility is mere speculation but nevertheless is posed as a question that can be approached experimentally If it can be verified it would provide an indication as to what might be expected if it were possible to initiate antibody formation or merely to alter the immunologic mechanism so that even if persistence of antibody is not demonstrated following artificial immunization with a so-called killed vaccine that the primary stimulation may act as a conditioning stimulus

The use of the term anamnestic reaction or recall reaction in describing the immunologic phenomenon associated with booster injections brings to mind the word memory It was for this reason that the chart in Figure 19 was prepared to suggest the analogy between the mechanism of convalescent immunity with that of memory and that both are a conditioning process The chart is self explanatory and that this phenomenon is operative for poliomyelitis as it is for other immunologic systems is illustrated in

TYPE 2 POLIOMYELITIS ANTIBODY RESPONSE IN VACCINATED HUMAN SUBJECTS

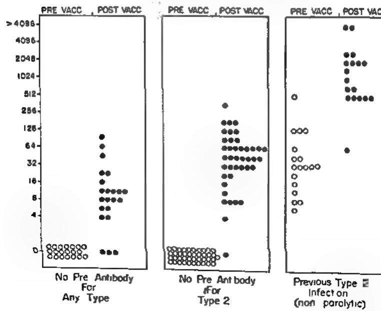


FIG 21

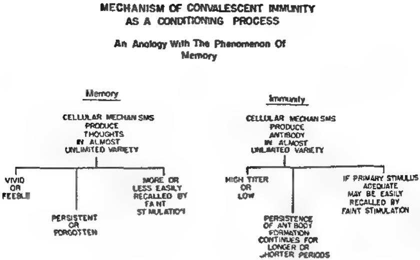

MECHANISM OF CONVALESCENT IMMUNITY
AS A CONDITIONING PROCESS
An Analogy With The Phenomenon Of Memory
Memory
CELLULAR MECHANISMS PRODUCE THOUGHTS IN ALMOST UNLIMITED VARIETY
VIVID OR FEEBLE
PERSISTENT OR FORGOTTEN
MORE OR LESS EASILY RECALLED BY FAINT STIMULATION
Immunity
CELLULAR MECHANISMS PRODUCE ANTIBODY IN ALMOST UNLIMITED VARIETY
HIGH TITER OR LOW
PERSISTENCE OF ANTIBODY FORMATION CONTINUES FOR LONGER OR SHORTER PERIODS
IF PRIMARY STIMULUS ADEQUATE MAY BE EASILY RECALLED BY FAINT STIMULATION

FIG 19

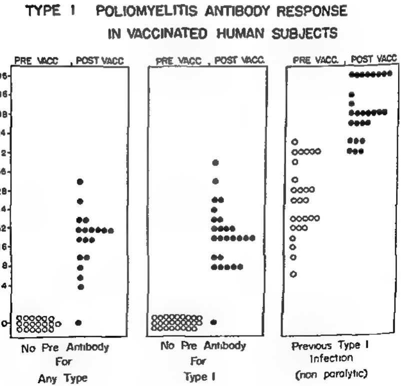

TYPE I POLIOMYELITIS ANTIBODY RESPONSE
IN VACCINATED HUMAN SUBJECTS
PRE VACC , POST VACC
>4096
4096
2048
1024
512
256
128
64
32
16
8
4
0
No Pre Antibody For Any Type
No Pre Antibody For Type I
Previous Type I Infection (non paralytic)

FIG 20

If in more extensive studies under way this is further substantiated then it may not be necessary to have to resort to measures requiring the use of immunologic adjuvants for the control of poliomyelitis. The proper adjustment of dosage using virus produced efficiently and rendered non infectious in as gentle a manner as possible may well provide the means for inducing the levels of antibody required. Further studies will indicate the way in which the booster phenomenon may be employed most effectively.

Concluding Remarks

It has been the purpose of this presentation to illustrate in a somewhat schematic fashion the mechanisms of convalescent immunity for a variety of diseases and to derive therefrom clues as to how certain of the infectious diseases that are still important public health problems may be controlled. The mechanism whereby convalescent immunity may be simulated for influenza and poliomyelitis by non infectious vaccines has been discussed in detail. Many hypothetical questions have been raised and answers to these will be had in investigations still to be performed. Nevertheless the direction of these studies and the theoretical basis for the approach to the solution of the remaining problems in the control of certain of the infectious diseases have been presented.

Figures 20 21 22 Here it may be seen that in persons who have had a previous immunologic experience with the types 1 2 or 3 poliomyelitis virus and in whom this experience was had as a result of an earlier non paralytic infection vaccination with a non infectious vaccine evokes a sharp rise in antibody to levels beyond that observed before vaccination On the other hand in persons who before vaccination have no antibody whatever either for any type or merely for the respective types and in the latter instance in such individuals there may have been antibody for one or both of the other types that the primary stimulation raised antibody titers to lower levels than was observed in persons who have had a prior immunologic experience The question that remains is to determine whether or not persons in whom antibody was initiated artificially by means of a non infectious vaccine will at a later date behave in the same manner as did persons in whom the first stimulus was in the course of natural infection If that is the case then it would appear that a simple means is at hand for raising antibody titers to high levels where they should be maintained for substantial periods of time and more than that be easily recalled by later faint stimulation This has already been shown in just a few human subjects and in large numbers of experimental animals

TYPE 3 POLIOMYELITIS ANTIBODY RESPONSE IN VACCINATED HUMAN SUBJECTS

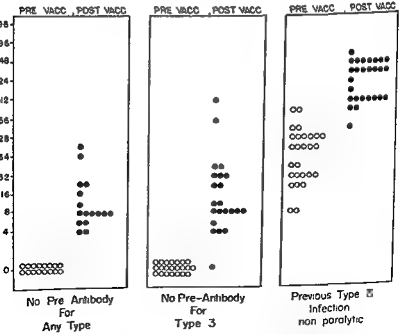


Fig 22

resembles closely the course of the infection in human beings with certain exceptions For studies in which large numbers of animals are necessary this species is used instead of the chimpanzee with due reservations regarding the carrying over of interpretations to the human disease

According to the schema illustrated in Figure 1 the disease is pictured as a sequence of stages of virus multiplication beginning somewhere in the alimentary tract spreading to lymphatic organs and in some instances spreading to the central nervous system (CNS) That virus multiplies in the alimentary tract and in the CNS is well known and its ability to reach lymphatic organs is beyond dispute since virus has been found repeatedly in the blood stream of children during the presymptomatic period by Horstmann[3] and by us The fact that virus can reach lymphatic organs by way of the blood of course does not constitute evidence for virus multiplication in such tissues Such multiplication is however strongly suggested by the recovery of virus from lymph nodes in both fatal human cases and in paralysed experimental animals after virus feeding when serum antibody is already present [4,5,6] Another reason for including these tissues in the schema is to ensure that an effort is made to establish that multiplication is responsible for the presence of virus in lymphatic organs rather than passive absorption of circulating virus It is interesting that hyperplastic changes have been described in lymphatic organs of autopsy cases for many years [7] and recently in a large series of cases by Sommers Wilson and Hartman at the Henry Ford Hospital [8] The significance of such findings has been in dispute throughout the years and careful virus studies of these tissues would

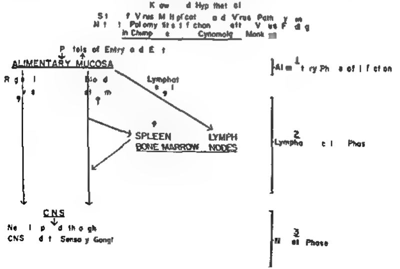


FIG 1

20

Sites of Immune Barriers in Poliomyelitis*

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The problems of immune barriers in virus infections are of course closely linked to problems of pathogenesis especially the sites of virus multiplication and the pathways of virus dissemination in the host Knowledge of both of these aspects of the pathogenesis of poliomyelitis as well as of mechanisms of induced immunity is incomplete so that our discussion will necessarily have to fill in gaps of information with the less substantial structure of speculation

Since certain types of information are not easily obtained from human infections Dr Howard Howe and I in 1939[1] adopted an anthropoid ape the chimpanzee as an experimental model which might resemble the human being sufficiently to make possible useful comparisons of basic infection processes Although the chimpanzee is at times an uncongenial collaborator each year has strengthened our admiration for its ability thusfar to do more than most models are expected to do namely to resemble the subject being modeled in every measurable way After virus feeding the observable phenomena of infection in chimpanzees such as fecal virus excretion viremia serum *antibody response clinical course and pathologic changes* are remarkably similar to the events of the natural infection in paralytic and in abortive human cases Moreover the finding of viremia and of a sharp antibody rise as regular events of the presymptomatic period in chimpanzees led us to seek and to find the same phenomena in household contacts of paralytic human cases[2] The developments of recent years moreover have also indicated that certain non anthropoid primates the cynomolgus monkeys are also very useful models for study of the pathogenesis of poliomyelitis After virus feeding in this species the course of the infection also

* Aided by a grant from the National Foundation for Infantile Paralysis Inc

Table 2

EXPERIMENTS SHOWING DIRECT PENETRATION OF NERVOUS SYSTEM OF CYNOMOLGUS MONKEYS BY VIRUS FROM BLOODSTREAM

Exp No		*Inoculation into Heart with 1 ml of Mahoney virus (Und to 10−)*		*Inoculation into Heart with Virus-contaminated needle (Undiluted virus)*
	Groups	*Paralytic Ratios*	*Inc Periods*	*Paralytic Ratios*
9 16 52	Virus alone	6/1	8 8 10 10 12 12	0/5
	Virus & rt calf injections	9/14	7 7 7 7 8 10 11 12 13	
3/3/53	Virus alone	5/10	8 9 9 11 13	0/5
	Virus & rt calf injections	16/20	8 8 8 8 8 8 8 9 10 10 10 10 11 11 13 14	
	TOTAL	36/54		0/10

matched with groups in which only a hypodermic needle was thrust into the heart *after its surface was dipped into undiluted virus suspension* It is clear that virus contamination of structures penetrated by the needle was not responsible for the infections in the groups inoculated into the blood stream

The median incubation period in cynomolgus monkeys with this virus is about 8 to 9 days after intravascular inoculation and after intracerebral inoculation but is 13 *days by intramuscular inoculation* and 12 *days after* virus feeding Virus therefore would seem to enter the CNS almost immediately from the blood stream since the incubation period by that route is almost as short as after direct intracerebral inoculation but considerably shorter than after virus feeding or intramuscular inoculation

In addition to the demonstrated ability of the virus under experimental conditions to travel to the CNS by way of the blood stream or by a direct neural route as after intranasal inoculations there is now strong evidence that a combination of these routes may be involved under special circumstances in both experimental animals and in human beings When viremia is induced in cynomolgus monkeys by intravascular inoculation of Mahoney virus and concomitantly an intramuscular injection is given in the right calf muscles with gelatin or with other materials including buffered saline it can be shown as we previously reported[10] that the paralytic rate is increased and localization of initial paralysis is reversed from preferential arm paralysis to preferential leg paralysis especially in the right leg Table 3 gives some of the evidence which establishes the reality of the provoking phenomenon under these conditions It should be kept in mind that the two essential experimental conditions namely viremia and intramuscular injection prevail in epidemic areas when intramuscular injections of penicillin

appear to be more hopeful for yielding convincing evidence of virus multiplication

Our schema is also speculative as regards the exact pathway of virus dissemination from peripheral sites of multiplication to the CNS The necessarily indirect evidence on this point has been reviewed before[9,10] and would require too much time for presentation here The evidence is clear enough in the experimental animal and in human beings that poliomyelitis virus has the ability to enter the blood stream as well as to travel along nerve fibers Which pathway to the CNS is actually utilized in human infections is not known It may be worth a few moments to present new evidence on this point In experiments with the Mahoney strain it was found that paralytic infection could be produced by intravascular inoculation of monkeys in dilutions equal to or higher than those capable of infection by the intracerebral route Table 1 shows comparisons of titrations by various routes of inoculation which indicate that this virulent strain is capable of initiating CNS infections from the blood in high dilution Even with large amounts of virus however the paralytic rate in many experiments has never exceeded 60% unless concurrent intramuscular injections of non viral materials were given It would appear that in this species the penetration of virus into the CNS from the blood stream wherever it occurs is limited not only by virus concentration but by as yet unknown constitutional factors in the host

Table 1

TITRATION OF MAHONEY TISSUE CULTURE VIRUS BY VARIOUS ROUTES IN CYNOMOLGUS MONKEYS (POOL TC 2 FROM MONKEY TESTIS)

Route of Inoculation	*Virus dilutions (Inoculum = 1 ml)*						*Endpoints Approximate*	
	Undiluted	*10^{-}*	*10^{-}*	*10^{-}*	*10^{-}*	*10^{-}*	*PD50*	*MID*
Tissue culture of monkey kidney			4/4*	4 8	1/8	0 8	10^{-}	10^{-}
Intrathalamic–cynomolgus‡		4 5†	3 5	0/5	0 5	0/5	10^{-}	10^{-}
Intramuscular–cynomolgus		2 5	3 5	1/5	1/5	1/5	10^{-}	10^{-}
Intravascular–cynomolgus	6/13	5 7	1/7	3/5	1 5		10^{-}	10^{-} or
Intravascular–rhesus	3/5	2/5	1/5	0/4	2/5		10^{-}	10^{-} or
Intravascular–cynomolgus (Cortisone injection calf muscles)	13 13	4/7	5/7	1/5	0/5		10^{-}	10^{-}

* Infected cultures/inoculated cultures

† Paralysed animals/inoculated animals

‡ All cynomolgus monkeys were of *Macaca irus* group from Cebu Philippine Islands

Table 2 presents data which support the idea that virus introduced into the blood stream actually invades the CNS by way of blood vessels In two experiments groups inoculated into the heart with 1 ml of virus were

CNS infection [11] The use of passive antibody eliminates the intrusion of other immune phenomena which for example might play a part in actively immunized animals The efficacy of low serum antibody levels fits the hypothesis of virus invasion from the blood stream after virus feeding Theoretically low serum antibody levels would also be sufficient to prevent the shunting of virus from blood to nerve fibers in instances where CNS invasion might be induced by intramuscular injections of non viral materials

Evidence for the level of antibody required to prevent invasion along nerves is inadequate Morgan s demonstration that active immunity against intracerebral challenge is correlated with high serum antibody levels did not throw much light on this point although it indicated an important role for serum antibody previously doubted by many [12] First of all the intracerebral route is extremely artificial and secondly Morgan s challenge doses of virus were very high I should like to present briefly two sets of my own experiments which test the role of passive serum antibody in preventing infection by neural routes when the virus is introduced peripherally rather than in the CNS In one set of experiments the challenge dose was given by a known neural route namely intranasally in rhesus monkeys This challenge is non traumatic Table 4 shows the results of intranasal challenge with a small dose in rhesus monkeys which had a moderate level of passive serum antibody (1 in 100) It is clear that after access to the nervous system is gained at the time of fever the passive antibody fails to influence the course of the infection as is well known from earlier work The impli

Table 4

THE EFFECT OF PASSIVE IMMUNOPROPHYLAXIS AND IMMUNOTHERAPY ON PARALYTIC RATE AND SEVERITY AFTER INTRANASAL INOCULATION IN RHESUS MONKEYS (10 PD50)

Summary of 3 Experiments†

Groups	Paralytic Rate*	Prostrating Paralysis	Fever	Incubation Period Days	Mean
Controls	16/18	12/16	17/18	7 7 7 8 8 8 9 9 9 9 10 10 10 11 11 NP	8 9
Treated on 1st day of fever	5/6	5/6	5/6	8 9,9 9 10	9 0
TOTAL	24/24	17/24	22/24		
Treated 1 hr or 24 hrs before inoculation	5/12	1/12	11/12	9 10 10 11 12	10 4

* Gamma globulin given intramuscularly in dose of 10 ml per kilogram in all treated animals Brunhilde virus used for inoculation on 2 successive days total of 1 ml of 1% suspension with intranasal titer of 10− and intracerebral titer of 10−

† Modified from Table 1[10]

‡ Negative animals had no lesions in olfactory bulbs One animal had non paralytic poliomyelitis

Table 3

INCREASED PARALYTIC RATE AND SELECTIVE LOCALIZATION OF PARALYSIS AFTER CONCOMITANT INTRAVASCULAR INOCULATION* AND GELATIN INJECTION IN RIGHT CALF MUSCLES

	Total of all Gelatin Expts		*Final Experiment*		
Paralytic Incidence	*Controls*	*Injected†*	*Controls*	*1 ml 1.5% gelatin*	*0.5 ml Procaine Penicillin*
Ratio	43/97	70/99	5/12	8/10	11/12
Per Cent	*44*	*69*	*41*	*80*	*91*
Localization of Initial Paralysis (1st day)					
Rt leg alone	4/43	31/70	0/5	6/8	3/11
Both legs alone	8/43	18/70	1/5	1/8	5/11
Total	12/43	49/70	1/5	7/8	8/11
Per Cent	*28*	*70*	*20*	*88*	*73*

* With 1 ml 10⁻ Mahoney Tissue Culture Virus

† 89 received 4 ml 1.5% gelatin

or of other materials are being given. Although there is no direct proof available, it seems reasonable that in instances where paralysis is the result of intramuscular injections the virus is shunted from injured blood vessels to injured nerve fibers in the injected muscle. This is particularly indicated since the localization of initial paralysis to the injected limb is characteristic of neural spread, as seen, for example, after direct inoculation into the sciatic nerve. We are testing this assumption more directly in experiments now in progress.

Immune Barriers and Virus Dissemination in the Host

In considering the problem of immune barriers I shall limit the discussion to mechanisms of induced immunity and also shall deal separately with barriers to virus pathways in the body and barriers to virus multiplication. In connection with virus dissemination the role of circulating antibody is of course paramount, especially the role it may play in blocking the pathway of virus to the central nervous system. From the foregoing discussion of pathogenesis, it appears that the job expected of immune barriers in preventing virus from reaching the CNS depends on the hypothesis selected as describing the route from the periphery. The efficiency of circulating antibody as a barrier could conceivably depend on the route of the virus in passage from the periphery to the CNS. Some evidence is available in one of our experimental models for the level of serum antibody which is effective in producing a barrier between the periphery and the CNS. Last year I described virus feeding experiments in cynomolgus monkeys in which very low levels of passive antibody were shown to be capable of preventing

muscular injections of gelatin a proved provoking agent under certain experimental conditions If it should be shown that gamma globulin is truly effective prophylactically for several weeks in the small doses employed in the human field trials this effect would have been achieved with levels of passive serum antibody not detectable or barely detectable by our present methods

Our chimpanzee experiments also suggest that serum antibody may impose barriers not only to CNS invasion by virus from the primary phase in the alimentary tract but also to invasion of antibody forming organs by such virus The evidence however is indirect and I present it as an intriguing problem which deserves further study As a result of infection after virus feeding in chimpanzees [16] in cynomolgus monkeys [17] or in human infections serum antibody rises sharply after the stage of viremia and approaches or reaches peak levels before the onset of paralysis as a rule The height of the antibody rise and its time course are identical in paralytic humans and in chimpanzees as well as in abortive cases in both species in whom viremia has previously occurred (Figure 2 and Table 5) The sharp antibody rise is also observed in asymptomatic infections in the chimpanzee in which it can be proved that CNS virus invasion has not

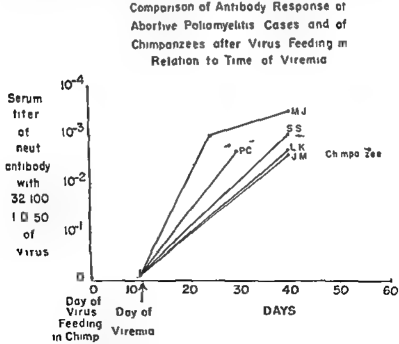

FIG 2

cation is plain that dissemination along nerve fibers within the CNS and across synaptic junctions is little if at all affected by this level of circulating antibody Moreover although there is a clear indication of protection when the antibody is present before inoculation the protection is not solid although the serum antibody level is about 100 times greater than in the virus feeding experiments previously mentioned In these experiments the virus is placed directly on nerve cells in the surface layer of the olfactory mucosa It can be argued alternatively either that the failure of complete protection is due to the fact that nerve cells are directly exposed unlike any other anatomical situation in the body or that the evidence of partial protection suggests an imperfect antibody barrier Conceivably this barrier is produced by the excretion of passive antibody in the nasal secretions comparable to that demonstrated by Bell in nasopharyngeal secretions [13] At any rate it is fair to ask whether as much antibody is required in other situations where virus conceivably may have to traverse antibody-containing tissue fluids before gaining access to nerve fibers Entirely satisfactory evidence is not available but some suggestive ideas on this point may be gained from a second set of experiments on passive protection against peripheral neural inoculation In these previously published experiments the challenge was given intramuscularly in the calf in rhesus monkeys with viruses which regularly produce initial paralysis in the corresponding leg [14] This route involves some trauma and the localization of initial paralysis implies that the virus gains the CNS along peripheral nerves from the inoculated muscle It was observed that a high degree of protection was obtained with a serum antibody level of only 1 in 32 when approximately 1 intramuscular PD50 was employed However since 1 intramuscular PD50 represents 100 000 intracerebral doses it can hardly be argued that not enough virus was used Moreover when the passive antibody level was allowed to fall over a period of 3 weeks resulting in a barely detectable serum antibody level some protection still appeared to be conferred against a similar intramuscular challenge Although these results suggest that even access to peripheral nerve fibers may be barred by low levels of antibody the artificiality of the route of inoculation unfortunately prohibits a close comparison with the oral route of infection After virus feeding for example the degree of primary virus multiplication in the alimentary tract may affect the ability of circulating antibody to bar access to nerve fibers In summary the evidence does not permit a precise statement regarding the site where serum antibody prevents access of virus to the CNS after experimental virus feeding but such access is prevented by low levels of circulating antibody It had been hoped that evidence would be available from the study by Hammon and his colleagues regarding the role of very low passive antibody levels produced by gamma globulin injections in preventing CNS invasion in human beings [15] It is plain from the evidence I mentioned earlier however that their results require confirmation since their control groups received intra

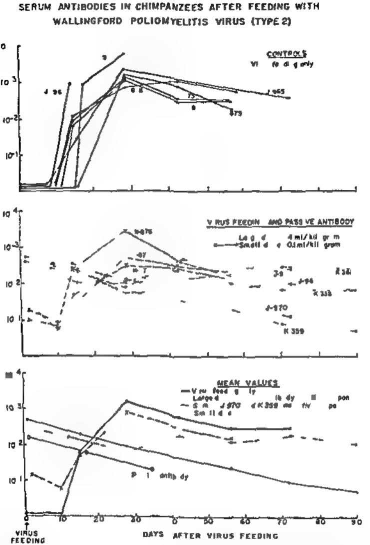

FIG 3 Experiments discussed on page 252 and described in detail in reference 17

tion wherever it may be in the alimentary tract it is clear for example that in animals recovered from a primary infection reinfection as evidenced by fecal virus excretion is usually prevented after challenge with the same virus or a homotypic virus [18,19,20] The presence of active serum antibody levels of about 1 in 100 to 1 in 500 does not seem to be responsible since

Table 5

TYPE 1 SERUM ANTIBODY AND VIREMIA IN HOUSEHOLD CHILD CONTACTS (1–13 YRS) OF PARALYTIC POLIOMYELITIS CASES*

		Converters			
	Immunes	*Antibody—1st bleeding*	*No antibody—1st bleeding*	*Escaped infection*	*TOTAL*
Serum Antibody—1st bleeding†	>1:10	1:4 to 1:50	<1:4	<1:4	
Serum Antibody—1 mo later	>1:10	1:200 to 1:300	>1:100	<1:4	
NUMBER	63	7	12	1	83
% of TOTAL	75.9	8.4	14.5	1.2	
Viremia at 1st bleeding	0	0	5	0	5
% with Viremia			42		6

* Rochester, Minn and Baltimore—1952 [30]

† Within 7 days of onset of index case

occurred [16] This establishes that the sharp early antibody rise is not the result of virus multiplication in the CNS It must be the result of virus multiplication in the alimentary tract or of virus multiplication or absorption *in the secondarily invaded lymphatic tissues* or of both However moderate levels of passive antibody which have no effect on fecal virus excretion inhibit the sharp early rise as shown in Figure 3, whereas low passive antibody levels either do not inhibit it or do so but slightly [17] An attractive but unproved interpretation of this finding is that a moderate level of antibody (1 in 100) prevents all virus spread to lymphatic organs whether by the blood or by lymph vessels and that the slow antibody rise *seen with such levels is due solely to antibody formation in the alimentary* wall itself and possibly in lymphatic tissue therein The low passive antibody level (1 in 32) however clearly is sufficient to bar virus from the blood stream and yet does not quite prevent the sharp early active antibody rise One may postulate that with such low levels virus may still escape from the alimentary wall to regional lymph nodes by way of the lymphatics but cannot reach a lymphatic organ such as the spleen because of the antibody barrier in the blood stream According to this view the high early antibody rise may be due to antibody formation in the regional lymph nodes alone or in such structures acting in concert with the spleen bone marrow or other lymphatic structures accessible via the blood stream

Immune Barriers at Sites of Virus Multiplication

Finally, it is necessary to consider those immune factors which act locally at the sites of virus multiplication and which have not been shown to be associated with circulating antibody In the primary site of virus multiplica

symptoms but it is not clear whether this drop in titer occurs intracellularly or is the result of neutralization of intracellular virus by local antibody during the process of preparation of the spinal cord suspension Figure 4 shows that the great accumulation of lymphocytes in the tissue which occurs with some virus strains during convalescence could well be the source of at least some of the antibody in the CNS in this period The apparent rapid subsidence of infection in the CNS is in striking contrast to the longer duration of infection in the lower alimentary tract resulting in fecal virus excretion for a period of weeks or longer As I mentioned earlier the course of the infection in neither site appears to be influenced by passive circulating antibody Since the exact site of virus multiplication is not known in the lower alimentary tract it is not possible to say whether this prolonged infection process is correlated with the absence of an inflammatory process such as occurs in the convalescent as well as the acute stage of infection in the CNS

Thus the demonstrated local immunity to reinfection of the alimentary tract and of the CNS remains wide open for further investigation Although their mechanisms are unknown it is interesting that only a few years ago

FIG 4 Rhesus B 190 35 days after onset of paralysis showing massive perivascular lymphocytic infiltrations containing germinal" centers in one anterior horn The other anterior horn has largely recovered from the infection and contains only negligible residual glial changes The magnitude of the lymphocytic response varies with different virus strains Gallocyanin stain × 25

passive serum antibody levels of the same magnitude do not appear to have the slightest effect on fecal virus excretion Yet these levels of antibody are 100-fold greater than those which appear readily to prevent spread from the alimentary phase to the neural phase [17] The resistance to alimentary reinfection is highly type specific which suggests a role for local antibody It is persistent for months after the apparent cessation of fecal virus excretion which possibly throws some doubt on the potential role of virus interference Beyond this the phenomenon has not had extended analysis although the role of alimentary infection in immunogenesis in the human population makes it very important to know the effect of various degrees of partial immunity on susceptibility to alimentary infection

Interestingly enough the problem of reinfection in the CNS has a number of analogies with the problem of reinfection in the alimentary tract A high degree of type specific immunity is produced by paralytic infection resulting from intracerebral challenge [21] yet the serum antibody level may be extremely low after paralysis due to intracerebral inoculation as shown by several investigators [2] [2] [3] An additional fact which speaks against any role of serum antibody in this type of immunity is that a surprising degree of cross or heterotypic immunity occurs after paralytic infection Although this cross immunity is observable as a reduction of the expected paralytic rate and of severity of paralysis after challenge of convalescents with another virus type the degree of protection of this kind is considerably less than the almost absolute resistance against homotypic challenge Yet when vaccinated animals with high serum antibody levels are challenged with heterotypic viruses very little or no protection is usually observed [4] [6]

The local immunity of the previously infected CNS is therefore unrelated to circulating antibody but little more can be said about its origin Locally formed antibody hardly seems to be the whole story since it does not explain the heterotypic immunity and since the levels of antibody in the CNS observed by Morgan were later shown by her to be largely accountable for by accumulation of circulating antibody rather than by local formation [7] The failure of Sabin and Steigman to confirm Morgan s finding with Type 1 strains of recent origin has not been explained [8] The difference in their results could conceivably be due to difference in the duration of persistence of different virus strains in the CNS or to the great variation of the lymphocytic response after infection with different virus strains This response may be prodigious in some cases or relatively slight in others and it would be surprising if this variation were not correlated with local accumulation or formation of antibody

The problem is open whether such locally formed antibody could account in part for the rapid subsidence of infection in the CNS in some instances perhaps by preventing spread of virus from nerve cell to nerve cell It is clear with Lansing virus infections in the monkey that demonstrable virus in the spinal cord falls exponentially after the second day of

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some of us at least considered this type of immunity to represent the central problem of immune barriers in poliomyelitis Now it is clear that circulating antibody is a potent force to be reckoned with and theoretically could be the critical factor in overall immunity to poliomyelitis Only time and con clusive field tests of the efficacy of low levels of serum antibody induced by passive immunization or active immunization with inactive vaccines can give us the answer *If this answer should perchance be in the negative we* may still have to fall back upon the local immune barriers induced by active immunization with live viruses

Table 6

SITES OF IMMUNE ACTION IN NATURAL POLIOMYELITIS INFECTION OR AFTER VIRUS FEEDING IN CHIMPANZEES AND CYNOMOLGUS MONKEYS

Sites of Antibody Formation	*Action of Pre Existing Serum Antibody*	*Action of Local Im mune Factors Induced by Prior Infection*
ALIMENTARY MUCOSA—Slow serum antibody re sponse (Poor access to cir *culating antibody)*	Moderate levels (1 100) do not prevent infection of lower alimentary tract Effect of high levels (> 1 1000) not known	Inhibits reinfection of alimentary tract by homotypic virus
LYMPHATIC ORGANS—Rapid serum antibody re sponse (Good access to cir culating antibody)	Low levels prevent vascu lar spread prevent rapid high response of spleen Moderate levels (1 100) prevent vascular & lym phatic spread prevent rapid high response of spleen or lymph nodes	No evidence Should inhibit homotypic virus *reinfection*
CNS—Slow serum antibody response (Poor access to circulating antibody)	High levels prevent initia tion of CNS infection after direct neural inocu *lation (1 1000)* Low levels prevent spread to CNS via blood (or peripheral nerves?)	Inhibits re infection of CNS by homotypi virus Some inhibition against heterotypic viruses

Table 6 summarizes the framework around which my discussion was built and for which I presented such evidence as seemed to be at hand Its principal purpose is to emphasize that the problem of over all immunity of the host is a complex one and will not be fully understood until we have a more complete knowledge of virus multiplication and dissemination on the one hand and of the mechanisms of local and of humoral immunity on the other

21

Mechanism of Active Induced Immunity with Attenuated Living Vaccines

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As Dr Salk has already pointed out there is a definite difference between the immunity produced by inactivated virus vaccines and that following convalescence from clinical disease in both the degree and duration of the immune state I think it is safe to say that in spite of adjuvants in general there is no immunity like convalescent immunity In fact for many years physicians have recognized this and on occasion in certain of the less dangerous virus diseases like mumps and measles have purposely exposed children at an optimal age and state of health thereby establishing a solid permanent immunity At the present time for instance the exposing of young girls to German measles is considered a perfectly justifiable procedure This is scarcely indicated in the more serious infectious diseases and is not exactly a recommended public health approach to preventive medicine In fact this procedure sometimes backfires One of our staff members tells how his son while in elementary school told him his playmate had just come down with mumps Knowing his son to be still susceptible and thinking of the orchitis he might get if he were to wait till after puberty to develop mumps he told his boy Why don t you go over to your friend s house and visit him? Sit on his bed and play cards to help him forget being sick The boy did as instructed but a few days later on talking to the playmate s parents the father got the horrifying news that the infection was a clear cut case of scarlet fever not mumps The end of the story is that the experimental exposure was successful

However there are other less drastic ways of accomplishing exposure to active infection with the subsequent development of a convalescent type of immunity We are all familiar with the use of immune gamma globulin to

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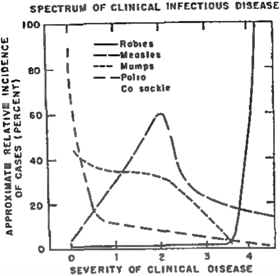

FIG 1 Distribution of cases of certain human virus diseases according to their clinical severity (0 severity indicates inapparent infections)

But what is the evidence that this statement is true? One of the first indicators is the fact that not all live virus *preparations* are in truth live virus *vaccines* Before we get too far perhaps a simple definition is in order I would say an attenuated live virus vaccine is one in which a relatively small number of viable virus particles given in a single dose can produce an immunity comparable to that in convalescence from the active disease but causing at the most only minimal evidence of clinical illness There is no good evidence that I know of that viruses have some special terrificly stepped up antigenicity merely because they are live when inoculated They are often shown to be more antigenic when living than when inactivated but live viruses when acting as non replicating chemical entities have no super antigenic qualities resulting in a permanent immunity This is readily demonstrated in the field of rabies vaccines Live virus rabies vaccines have been used for years yet until the recent development of the Flury attenuated chick embryo vaccine there was no demonstration of a longer lasting immunity in animals or of greater prophylactic efficacy in human rabies by these live virus vaccines than by the inactivated virus preparations

Practically all the available evidence points in the same direction—to be effective as a vaccine an attenuated live virus must multiply—must actually produce an infection in the host Practically every live virus vaccine being used effectively today is an example of this fact Obviously it is true in smallpox immunization—we have the visible proof that infection takes place—in yellow fever the frequent occurrence of fever after vaccination

effect a mild attack of measles and more recently to protect against infectious hepatitis and polio At least in the case of measles and hepatitis there is evidence of an active immunity resulting after this type of exposure to a modified natural infection

Yet from a practical standpoint the use of attenuated live virus vaccines is the most important method of purposeful exposure Although many people are still hesitant about embracing the concept of widespread use of live virus vaccine in a number of human virus diseases the fact remains that in the history of preventive medicine the outstanding successes of specific immunization in the virus disease field have been with live virus vaccines—namely in smallpox and yellow fever

Maybe you will agree with me that viruses like people are ambitious and are just as anxious to get along in the world as are virologists There is reason to think that viruses take the long range view of life—namely the survival of the species—and as pointed out by many others in the past this is not best accomplished by destroying all its susceptible hosts The best arrangement seems to be a symbiotic one in which both virus and host survive In support of this we are finding more and more infectious diseases to be caused by agents that give rise to latent infections in the majority of hosts with clinical disease the unusual occurrence in a few There are many examples of this in animals—mouse encephalitis lymphocytic choriomeningitis psittacosis and several in man such as herpes psittacosis hepatitis So we look on latency of infection often accompanied by immunity and always a possible source of spread of infection as probably a much more common phenomenon than we now have definite evidence for

But even in those virus infections where there is no reason to suspect a persisting latent infection but rather a short lived association of virus and host with subsequent immunity and apparent clearing of virus from the host we have further evidence of the reasonableness of these agents in the broad spectrum of the clinical disease they produce Figure 1 is an attempt to indicate graphically the approximate distribution of clinical manifestations of a few virus diseases

Here we have begun to use the terms inapparent infections as compared to latent infections and it is obvious that Nature without the help of chick embryos or tissue culture is doing a pretty good job of painlessly producing a permanent type of immunity There is no new (and perhaps dangerous) principle being laid down by man in the use of attenuated live virus vaccines the principle was probably laid down at the time the first virus broke off the first gene or wherever this conference decides viruses probably came from

Therefore what we are talking about when we speak of the mechanism of active induced immunity by attenuated live virus vaccines is the same thing as active immunity following infection—especially the so-called inapparent infections

lation of antiserum does not prevent the subsequent development of an active immunity This I believe can probably be explained on the basis of the relative speed of effective adsorption and distribution of antibody and virus Much experimental evidence points to the rapid distribution of inoculated virus and once it has reached susceptible cells their even more rapid attachment and invasion Passive serum antibody is not adsorbed from the site of inoculation so quickly In a recent study we have investigated the adsorption of antirabies horse serum in humans and have found more evidence of antibody in the blood 3 days following intramuscular inoculation than at 1 day It is interesting to note here also that in the same study the administration of a course of inactivated rabies vaccine after a dose of antiserum had no effect on serum neutralizing antibody levels derived from the passive antibody Neither did the presence of passive antibody interfere with the subsequent active antibody response to the vaccine

Thus there is ample evidence for what is an obvious conclusion—that true attenuated live virus vaccines result in virus multiplication in the host But the next logical and more fundamental question is not so easy to answer —why do they not cause clinical disease? Certainly there are two factors operating in virus infections the combination of which determines the extent of cell damage or dysfunction and therefore the extent of clinical illness This can be due to change in the virus itself which is the basis for most attentuated virus vaccines such as for hog cholera distemper and yellow fever Or it may be due to properties of the host as in the use of virulent infectious bronchitis virus to immunize chickens Certainly in epidemics of polio we frequently see the importance of the host factor which determines inapparent infection in many and severe paralytic disease in the few all seemingly infected with the same virus

The importance of the host is seen in the technics that are used to develop these attentuated virus strains Almost invariably they have been adapted to and carried often for prolonged passages through an unnatural host Frequently in the early stages of this adaptation difficulty is encountered and often the blind passage technic must be used This merely means that here on the opposite end of the production line when starting out to make an attenuated virus vaccine inapparent infections are also encountered in the unnatural host At the other end when the vaccine is used in practice usually the virus is then well adapted to the originally unnatural host but now the animal receiving the vaccine has become the unnatural host So the so-called natural resistance of the host to his old virus friend who has changed so drastically since the last time they met that he no longer recognizes him is put to advantage And I believe this lack of recognition is because the attenuated strain is really a different virus from the original pathogenic one—just as different as the naturally occurring variant is In fact it is so different that if you want to change it back to the virulent form you have to work at it by repeated passage in the original host That this

suggests it and the demonstration of actual virus in the blood at that time clinches the argument The same is true of the newer vaccines like the rabbit strain of hog cholera the Blacksburg Newcastle virus chick embryo distemper virus mouse and chick embryo dengue vaccine, and the latest—Dr Koprowski s TN strain of Lansing polio—in every instance virus multiplication in the vaccinated host can be demonstrated by virus isolation and titration The only exception I can think of is the Flury rabies vaccine now used in dogs So far all attempts to show the presence or multiplication of this attenuated virus in vaccinated animals have been negative but even here we have indirect evidence that the virus multiplies Table 1, from the

Table 1

COMPARATIVE DETERMINATIONS OF POTENCY OF HIGH EGG PASSAGE FLURY RABIES VIRUS VACCINE IN MICE AND GUINEA PIGS IN RELATION TO INFECTIVITY TITER IN SUCKLING MICE

Egg Passage	*Animal Species*	*Infectivity titer in baby mice (M–B) and mortality Ratio of adult mice (M–A) and guinea pigs (GP) challenged* after immunization† with dilutions of Flury virus*						
		1 0	2 0	3 0	4 0	5 0	6 0	
187	M–B		6/6	6/6	2/6	0/5	0/6	
	M–A	0/6	0/6	0/6	4/5	6/6	6/6	
	GP		0/6	2/6	6/6			
		0 70	1 40	2 10	2 80	3 50	4 70	4 90
197	M–B	5/5	5/5	6/6	6/6	5/5	5/5	2/6
	M–A	0/5	0/6	0/6	0/6	0/6	1/4	
	GP			0 6	0 6	0/6	0/6	4/6

* Three weeks after immunization

† Mice injected intracerebrally guinea pigs injected intramuscularly

NOTE Dilutions are expressed as logarithms to the base 10

work of Koprowski shows that a titration of the immunizing capacity of this vaccine parallels its infectivity titer This correlation it seems to me can be explained only on the basis of multiplication of the attentuated virus even though it cannot be demonstrated as such

An important point in the evidence for multiplication of these attenuated viruses used as vaccines would be the demonstration that their immunizing capacity can be neutralized by the presence of specific antibody When directly mixed with antiserum this is usually the case but when antiserum is administered to the test animal at a site different from that in which the vaccine is given there is not such uniform evidence of neutralization Inhibition of a primary take with vaccinia virus when anti vaccinia serum is administered simultaneously has been reported However with live virus vaccines against hog cholera rabies and distemper the simultaneous inocu

any organ In tissue culture studies it is not infrequent to see some areas of cells completely destroyed while adjacent ones remain viable or are involved at a later period An interesting point along this line is the evidence that as expected most organs have a tremendous reserve of cells and a high proportion must be incapacitated before clinical symptoms appear In polio some workers feel that up to 60% of the anterior horn cells can be destroyed before clinical paralysis develops However I believe the explanation based on the relatively smaller quantities of new virus is on a more fundamental basis There is some evidence that the growth cycle of these attenuated strains is more prolonged and it is interesting to speculate on the basis of evidence from bacteriophage that a certain intracellular concentration of virus particles is necessary before destruction of cells takes place This level may not be reached with attenuated strains

Unfortunately very little histopathology has been done on the tissues of vaccinated animals supporting the multiplication of these attenuated viruses However in some instances just as in the inapparent infection with natural viruses there are cellular reactions qualitatively similar to those in disease but quantitatively less

Further interest in the intracellular behavior of these agents revolves about the site of their multiplication—does it differ from the virulent strain? We have attempted to determine this with rabies virus Extensive studies have shown that at all stages of rabies infection in mice after intracerebral inoculation of a virulent fixed strain virus was most associated with mitochondria High egg passage of Flury strain of rabies when inoculated intracerebrally in adult mice produces an inapparent infection and by blind harvest after 5 days can be passed serially in adults However it can be demonstrated and quantitated only by intracerebral inoculation of suckling mice In Table 2 are the results of titrations of subcellular fractions from mice down with symptoms after virulent virus compared to those from mice having an inapparent infection after attenuated vaccine virus Here again we see the relative inefficiency of virus production with the attenuated strain

Table 2

SUBCELLULAR DISTRIBUTION OF ADAPTED AND ATTENUATED RABIES STRAINS IN MICE

	Titers of brain cell fractions				
	Hom	*Nucl*	*Mito*	*M c o*	*Sup*
Mouse passage fixed virus*	5 6	5 0	6 5	4 4	5 3
Adult mouse† passage HP Flury	9	2 5	>3 4	5	1 6

* Harvested 3 days after IC inoc —Titration IC in adult mice

† Harvested blindly 5 days after IC inoc —Titration IC in 5 day old mice

difference is not just a biological whim on the part of the virus is indicated in some recent results in the plant virus field suggesting that the chemical composition of an avirulent variant differs from that of the virulent parent strain This natural resistance of the host may be exploited by selecting the age at which resistance is high as is done with infectious bronchitis of chickens inoculating a virus which would cause a high mortality in baby chicks, but when used in 8 to 16 week old birds creates an epidemic of mild disease before the egg productive age

Also a factor in natural resistance of the host is the frequent finding by all virologists that not all the tissues of an animal are equally capable of supporting virus growth or if the virus does multiply equally susceptible of being damaged by it This is taken advantage of in smallpox vaccination by the percutaneous introduction of vaccinia virus and in the immunization of chickens against fowl pox and tracheobronchitis by virus strains which if introduced by the respiratory route would cause clinical disease

But this now raises the question of where these attenuated viruses do their multiplying Most of them are introduced either subcutaneously or intramuscularly yet there is no evidence that they stay localized or necessarily multiply in subcutaneous tissues or muscle Actually there is evidence that at least some of them multiply in the same tissues that are invaded in the natural disease Thus attenuated hog cholera and distemper vaccine viruses can be most easily recovered from the spleens of vaccinated animals just as the virulent viruses can be best isolated from this same organ in clinically ill animals Chick embryo attenuated Newcastle virus is administered intranasally and multiplies in the respiratory tract In fact after this procedure contact vaccination of other susceptible birds occasionally takes place through the natural mode of transmission Certainly vaccinia virus and 17D yellow fever virus get distributed to highly susceptible tissues via the blood stream

So we are faced with the fact that these viruses in most instances do reach invade and multiply in those susceptible cells usually involved in natural infection—but still no clinical disease Here I believe it is necessary to examine the quantitative aspects of the multiplication of these attenuated viruses Just as in latent infections here also new virus production is definitely less in amount and frequently at a slower rate than seen in acute natural disease The spleens of hogs after attenuated hog cholera vaccine have a demonstrable virus titer several logs lower than those with clinical cholera and reach that titer at 7 days by which time animals receiving virulent virus would be dead of the disease Bang and his coworkers have shown this also to be true of the vaccine strain of Newcastle virus compared to a virulent strain Whether this lower level of virus replication means that less virus is produced per cell or less cells are involved is not apparent Certainly the same spectrum of clinical disease from inapparent to severe also must be reflected in the relative susceptibilities of the multiple cells of

canine hepatitis the mammary gland in Q fever and the respiratory tract in psittacosis These examples are evidence only in *favor* of tissue immunity There are many on the other side of the fence Nevertheless the local production and release of attenuated virus antigen in the area of the susceptible cells even though in relatively small amounts may be quite effective in producing local tissue immunity

Now to return to the rate of multiplication of these attenuated viruses let us look at it in relation to the production of antibodies That serum antibodies are important in immunity to virus diseases especially those involving a viremic stage is well demonstrated by experimental and epidemiological data If there ever needed to be a clinching argument on this point we have it in recently reported cases of complete absence of gamma globulin in certain children and their repeated infections with such a virus disease as mumps Likewise serum antibodies may be important in determining if an infection with attenuated virus will be inapparent or clinical The slow rate of multiplication of these strains and in consequence the slowness with which they spread from cell to cell before enough are involved to cause clinical symptoms may very well give ample time for active antibody production If serum antibody could thus catch up to and overtake virus multiplication and spread clinical disease might thus be prevented but immunity established As indicated in Figure 2 taken from the work of Overman in our laboratory there is some evidence that this may be true Working with a strain of mumps virus adapted to intracerebral infection of suckling hamsters Overman compared the virus growth curve in the brain and the H I antibody titers in serum in relation to the development of clinical encephalitis and death in animals of different ages These results certainly suggest that the appearance of antibodies at a time before virus multiplication had reached too high a level could have been the factor preventing the development of disease and death It is interesting to note here that in natural infection with mumps virus in man we found evidence that the rapidity of serum antibody response during the incubation period of the disease appeared to be a determining factor in the spread of virus to other glands such as the testicle

I suppose no discussion of the mechanism of immunity after inoculation of attenuated live virus vaccines would be complete without consideration of the possible role of the phenomenon known as interference That this experimental procedure can bestow protection to animals is well recognized but the necessity for large amounts of the interfering agent and the time relationships required for its effectiveness are potent arguments against this as the mechanism of immunity produced by attenuated virus vaccines However in two other aspects of the use of these vaccines interference may be important First the innocuousness of the vaccine virus may be dependent upon the fact that the overwhelming preponderance of attenuated virus particles may prevent by interference the invasion and multiplication of

but no evidence that the intracellular site of its multiplication differs from that of the virulent strain

The site of virus multiplication and subsequent release of antigen may well play an important role in determining the subsequent immunity of the vaccinated animal The question of the existence of so called tissue immunity and its relation to humoral immunity is quite pertinent to this discussion I believe a consideration of this subject is a little less complex when we confine ourselves to those virus infections in which a viremia is not a necessary step in the pathogenesis such as in rabies or some respiratory disease I would like to cite some experiments we did several years back in which mice were immunized with a given dose of ultraviolet inactivated rabies vaccine by repeated intraperitoneal inoculation in one group and by intracerebral inoculation in a second group On subsequent intracerebral challenge those immunized intracerebrally were more immune than those which received vaccine intraperitoneally Furthermore the perfused brains of mice from each group were emulsified and tested for their ability to neutralize standard virus with which they were mixed Brain emulsions from intraperitoneal vaccinated mice did not neutralize at all while those from intracerebral vaccinated animals neutralized 100 MLD of virus This was true in spite of equal serum neutralizing antibody titers in the two groups Recent experiments by Komarov using a chicken embryo adapted rabies vaccine virus which causes inapparent infection after IC inoculation of adult mice showed these mice to be subsequently immune to all concentrations of the most virulent strain of virus by intracerebral challenge Also with attenuated influenza viruses given intranasally in mice or ferrets and with Blacksburg egg strain of Newcastle virus intranasally in baby chicks there has been evidence of an enhanced immunity compared to intramuscular inoculation and in the latter instance this occurs whether or not the baby chick has serum antibody received through the yolk of the eg at time of hatching

In a consideration of tissue immunity and its persisting effectiveness in protecting the host against infection it is important to distinguish between those cells which normally are being replaced such as the epithelial cells of the respiratory tract and cells which are not replaced such as nervous tissue ganglion cells Any change in cells as a result of a nondestructive infection may not be passed on to daughter cells and the precursor types of cells may not have been involved at the time of original infection At least it is convenient to use this as the explanation of why convalescent immunity after some respiratory virus infections does not persist This has also been suggested as the mechanism responsible for persistence of latent virus infections in spite of the presence of serum antibodies where intracellular virus might be passed to daughter cells by cell division without ever being exposed to circulating extracellular antibody It is true that latency frequently involves cells undergoing repeated cell division as in the kidney in

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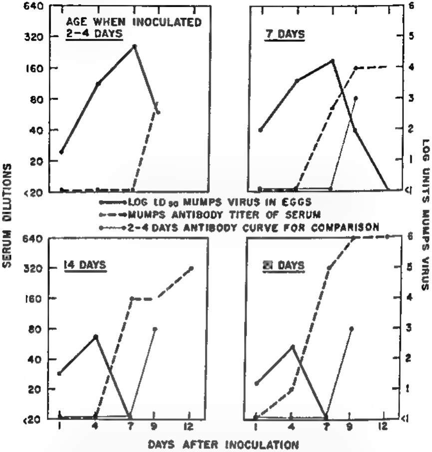

FIG 2 Rapidity of virus multiplication in the brain and serum HI antibody development in hamsters of various ages after receiving hamster adapted mumps virus IC (Hamsters 2 to 4 days of age die of mumps encephalitis at approximately 9 days but older hamsters remain healthy)

From Overman and Kilbam 'The Inter Relation of Age Immune Response and Susceptibility to Mumps in Hamsters *J Immunol* 71 5 Nov 1953

the few pathogenic particles still included in the total virus population of the vaccine Also it has been shown by the group of workers at Lederle that immunity to challenge with virulent virus develops as early as three days after hog cholera vaccine and even at 1 day after distemper vaccine This very early immunity may well be on the basis of an interference phenomenon

In summary it appears that the mechanism of action of attenuated live virus vaccines is the same as that involved in the common occurrence of inapparent infections in Nature The factors responsible for an infection being inapparent include both the virus and the host but in those induced by attenuated virus vaccines changes in the virus are the more important

By adaptation to an unnatural host these viruses have a reduced capacity to multiply in the vaccinated animal or man Virus concentration per cell may be at a level too low to cause cell destruction spread from cell to cell may be so slow that serum antibody becomes a limiting factor or too few cells are destroyed to result in clinical disease

Let us again look at Figure 1 showing the spectrum of clinical disease From this it would appear that relatively little change in polio virus should be necessary to make it a good attenuated virus vaccine and this would seem to be the case with the Lansing type as I m sure Dr Koprowski will soon tell us On the other hand from this chart one would expect it to be a pretty tough job getting such a vaccine for rabies Yet actually of all the diseases here represented rabies is the only one for which a practical live virus vaccine is in use But it did take a lot of doing to make it—not until the 176th-egg passage in Dr Koprowski s laboratory did it completely lose its pathogenicity for cattle Again look at the chart and consider that with these vaccines we have pushed the curve way over to the left But let us remember it may not have been pushed completely off the page In other words in some few individuals in certain physiological states as occurred in some animals receiving attenuated virus vaccines the infection produced may not be so inapparent but this would be seen if at all only in an occa sional individual when very large numbers were vaccinated

22

Practical Application of Living Virus Vaccines

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Although the title of my communication is Practical Application of Living Virus Vaccines I shall purposely avoid a detailed description of the actual number of different types of vaccines available for practical use nor do I intend to bother you with figures showing the numbers of animals of varied species subjected to immunization procedures with these vaccines throughout the world This information might appropriately be supplied by manufacturers with each vial of the respective vaccines but is not particularly relevant to the topic *The Dynamics of Viral Infection*

The purpose of this paper is to review some of our knowledge concerning incentives leading in the past to the preparation and subsequent acceptance of living virus vaccines It is hoped that illumination of often forgotten facts may help to dispel the incontestable fog which has accumulated about some vital points and has prophylactically prevented some scientists from preserving their sense of proportion while examining the merits and demerits of live virus vaccines The motto of my talk will be a statement by Lord Mildew in a British trial of Dogget versus Port of London Authority There is no precedent for anything until it has been done for the first time *

There are 13 species of viruses known to be used on a sufficiently large scale in the practice of either human or veterinary preventive medicine for assemblage under the heading of living virus vaccines In alphabetical order these are African horse sickness blue tongue disease of sheep canine distemper fowl pox hog cholera infectious bronchitis laryngotracheitis pigeon pox pneumoencephalitis (Newcastle disease) rabies rinderpest vaccinia and yellow fever Two of these are used for prophylactic purposes in man yellow fever and vaccinia the others are employed for the immunization of animals In addition to these viral vaccines there are a few other

* Herbert A P *Uncommon Law* Methuen & Co Ltd London 1936

viruses which were and are employed in live form as an experimental procedure for the prophylactic immunization of men and animals These are (again in alphabetical order) Colorado tick fever[1] dengue infectious hepatitis[3] influenza[4 6] mumps[7 8] poliomyelitis[9 10] and Rift Valley fever[11 1] I am quite certain that I have not exhausted the list and I shall be happy to have the participants in this symposium enlarge it later by quoting their own experience

History of Living Virus Vaccines

The search which resulted in the discovery of living virus as a method of immunization was of course prompted by the fact that in only a few viral diseases is it possible to immunize with properly inactivated agents Even then the resulting immunity may be transient and the engendered protection may fall short of the mark set by the enduring immunity which follows convalescence from a natural disease Moreover certain viruses either because of their nature or because of the small concentrations of antigenic material available prior to inactivation cannot be inactivated for immunization purposes

Four viruses whose history and some of whose characteristics are traced in Table 1 fall in the latter category although perhaps traces of antigenicity may be found in preparations containing inactivated Newcastle disease virus

Table 1

FOUR EXAMPLES OF THE USE OF MODIFIED LIVING VIRUSES FOR VACCINATION PURPOSES

Disease	*History of the Attenuated Strain*		*Duration of Immunity Following Vaccination*
	Origin	*Source*	
Variola	Nature	Heterologous Host (Cowpox)	4 to 7 years
Newcastle	Nature	Homologous Host	Lifelong (?)
Yellow Fever	Laboratory	Tissue Culture	> 10 years
African Horse Sickness	Laboratory	Mouse Brain	8 years

I believe we are all in agreement that the discovery of the immunizing properties of cowpox in man constituted the first conscious application of the use of modified living virus for immunization purposes This discovery stimulated several generations of scientists to search for other modified strains of viruses You may note that cowpox was not man made Nature provided an agent which was recovered from a ruminant host and was found to be nonpathogenic for man Its remarkable antigenicity prompted Jenner to use it in the prophylaxis of smallpox Later on numerous further

modifications of vaccinia virus were initiated by man in the laboratory but the original variant was as safe as the man made mutants

Nature also provided the modified virus for vaccination of fowl against Newcastle disease In contrast to vaccinia the immunizing agent was recovered from the natural host—the chicken a species which may be decimated by an outbreak of Newcastle disease Of the two variants used at present for vaccination purposes one strain when administered intranasally to one day old chicks invokes local active immunity very often superimposed upon congenital passive immunity [15] The second variant is applied 12 to 13 weeks later and induces a systemic type of immunity operative during the life of the bird

In contrast to vaccinia and Newcastle disease the viruses of yellow fever and African horse sickness were modified by man Modification in both cases was accomplished in a gradual fashion Propagation in unnatural hosts mouse embryo and chick embryo tissue cultures for yellow fever virus [14 1] and mouse brain for African horse sickness [illegible] led the virulent strains to shed their pathogenic properties and to be incorporated into antigenically powerful vaccines

Duration of Immunity

Vaccination with living virus vaccines engenders a state of resistance which in many instances parallels that induced by a natural attack of illness without ever surpassing it unless a latent infection may occur

Duration of immunity against smallpox will not be dealt with since it was amply discussed by Dr Downie at this morning s session The vaccination of birds against Newcastle disease at 12–13 weeks of age protects them during their lifetime People immunized against yellow fever with the 17D strain more than nine years ago have been found to have circulating homologous antibodies at [illegible] concentration similar to that immediately following vaccination [17] and it is reasonable to expect that a state of resistance may remain operative throughout the lifetime of the vaccinated individual Immunity engendered by the African horse sickness live virus vaccines has been proved to persist in horses for eight years or longer [illegible] However in endemic areas yearly revaccination of animals [illegible] recommended because of the plurality of immunologically distinct strains By means of annual vaccinations solid immunity against minor antigens is induced and the range of protection considerably broadened [18]

Since there are no active virus vaccines available for these four diseases it is impossible to compare the duration of immunity induced by live virus vaccines to that engendered by inactive viruses

However in the case of rabies virus vaccines containing inactivated virus were known to display immunogenic power Comparative data on duration of immunity following vaccination of dogs with the two types of vaccine are summarized in the next table

Table 2

DURATION OF IMMUNITY OF DOGS FOLLOWING VACCINATION WITH SEVERAL TYPES OF RABIES VACCINE BY DIFFERENT INVESTIGATORS

Author	*Vaccine*	*Months between vaccination and challenge*	*Mortality Ratio*	
			Vaccinates	*Controls*
Johnson	Phenolized	1	0/25	17/25
		12*	6/5	41/5
Koprowski	Phenolized	12	3/22	18/25
		24	8/19	21/23
	Avianized	12	0/25	18/ 5
		24	3/25	21/23
U.S.P.H.S.† in co operation with Lederle	Phenolized	24	0/30	18/33
		39	8/34	31/36
	Ultraviolet	4	0/31	18/33
		39	7/30	31/36
	Benzenene Extract	4	0/30	18/33
	Avianized	4	0/33	18/33
		39	0/30	31/36

* 3 injections

† Preliminary results study not yet completed

Table 2 gives results of experiments conducted by Dr Johnson[19] and myself [10] Groups of dogs were vaccinated in the first experiment by Johnson with phenolized rabies vaccine These dogs were challenged with street virus one year later and six out of 52 died of infection Similar results were obtained with phenolized vaccine in experiments conducted by Mr Black and myself In contrast to killed virus the live virus vaccine (avianized) showed significant superiority over the inactivated preparations particularly in resistance to challenge two years after vaccination Table 2 also gives preliminary results of another comparative study as yet unpublished conducted along similar lines by Doctor Tierkel *et al* of the U S P H S * in cooperation with our laboratory Duration of immunity in dogs was tested at 24 and at 39 months following immunization with four different types of vaccines The results of the 24 month challenge study indicated equally high

* Preliminary results of this work were reported by Dr E S Tierkel and Dr M M Kaplan at the Fifteenth International Congress of Veterinary Medicine Stockholm 1953 and were published by Dr J H Steele in *Veterinary Medicine* 48 4?5–4 6 October 1953

efficacy of the vaccines in the face of a mortality ratio of 18/33 in the control animals but with challenge performed three years and three months after vaccination the live virus showed significant superiority over vaccines containing inactivated virus One may add here that the live virus competed against overwhelming odds since 68 years of research experience in the field of rabies has produced methods of virus inactivation resulting in vaccines of greater immunogenic power than in any other known virus infection

Some data Table 3 are also now available on the persistence of neutralizing antibodies following oral administration of live TN strain of poliomyelitis to man [1] Sera of nine out of fourteen immunized individuals were devoid of neutralizing antibodies against any type of poliomyelitis prior to feeding with TN strain

Table 3

PERSISTENCE OF NEUTRALIZING ANTIBODIES AGAINST TYPE POLIOMYELITIS VIRUS IN SERA OF VOLUNTEERS FED TN STRAIN

Individual No.	Oral Administration of virus: No. of Feedings	Oral Administration of virus: Months between 1st and last feed	Neutralization Type No.	Maximum serum dilution, months after oral administration of virus: 0	1	2	3	4	11	12	1	15	18	20	26	27	[illegible]	39	33	34	36
2	2	1	I	1 6	1 22	1 90															
			II			1 74								1 636							
			III			1 2 0															1 640
3	3	2	I	<1	1 15		1 75														
			II	<1 2									1 39								
			III										1 116							1 100	
4	3	3	I	< 2	1 38		1 141														
			II				1 3						1 31								
			III										1 3							1 [illegible]	
5	3	3	I		1 514		1 50														
			II	1 4			1 574						1 8								
			III										1 98							1 60	
6	2	2	I	< 2	00			1 200													
			II	<1 2				1 600						1 21							
7	2	2	I	1 3	1 30																
			II				1 100					1 39									
			[illegible]															1 90			
8	2	2	I	1 3	1 00	1 2															
			II	3			1 00		1 15												
9	2	2	I	<1 2	1 2		1 3 0														
			II				290				1 75										
			III								380						1 3 0				
10	1		I	< 2	1 111																
			II	<1 2	1 6																
			III	1 2						1 200				1 80							
12	2	3	I	<1																	
			II	<1 2	1 332											00					1
			III	<1 2												0					
13	1		I	< 2	1 3																
			II	<1 2	1 14					20											
			III	<1 2					1							1 60					
1[?]	1		I	15	1 3																
			II	2	72																
			III	1 7	00										1 7						
16	4	10	I	< 2		1 2 8															
			II	<1 2					9									1 60			
17	3	8	I	<1 2	1 200																
			II	<1 2		1 24							1 200								
			III										1						1 94		

These individuals were found to be immune to Type I virus.

† This individual was found to be immune to Type III virus

You may observe from data summarized in the table that the homotypic antibody level remained unchanged in the blood of all individuals with the possible exception of No 8 for three years and more after original exposure to the virus It is reasonable to assume that the immunity thus acquired may last beyond the three year period investigated in this study I may fore warn you that the above results were obtained by the mouse neutralization test—a method now antiquated and forsaken Anticipating Dr Bodian s question I hasten to add that the results of this obsolete test were con firmed by the ultramodern tissue culture neutralization test

I regret to say that it is impossible to compare the duration of immunity induced by living poliomyelitis virus in man to that engendered by inactive virus since there are no data available One would expect to find a similar study conducted with inactive virus vaccines before a mass immunization program is initiated What we do find is the exact opposite

Since we are done with speaking of duration of immunity problems it might perhaps be well now to discuss the proper and obvious safeguards we should use in deciding that this or that virus is sufficiently attenuated to be employed in a vaccine

Necessary Characteristics of an Attenuated Strain of Virus to be Used for Vaccination Purposes

The presence of sufficient living virus in a final product to meet minimum titer requirements From a practical standpoint the quantity of viable virus present in the vaccine at the time of its administration is of paramount im portance Since as Dr Habel pointed out to be effective as a vaccine an attenuated live virus must multiply we have to facilitate its invasion into the organism by supplying adequate amounts of infectious units The quite astonishing theory has sometimes been expounded that one infectious dose —since the agent is a living one—should suffice to start a chain of reac tions leading ultimately to immunity However as Dr Habel told you dif ferent sites may be involved in the multiplication of an attenuated virus as compared to its virulent forefather The original host becomes an unnatural host for the attenuated virus and a relatively large amount of virus may be required to overcome the threshold of resistance

The living attenuated virus also has to be resistant to adverse external influences such as high temperatures humidity exposures to light etc Vac cines prepared in the United States have to retain enough living virus when shipped to Tombouctou to perform effectively under local conditions of storage Attenuated viruses which are not hardy in character cannot be used successfully in living virus vaccines

Absence of other types of live viruses in the final product Although this point is obvious to anyone familiar with the work conducted in a virus laboratory I am not certain that mere cognizance of the danger will solve the problem Proper and obvious safeguards should be taken at the time

when such vaccines are produced and adequate tests should be made before the final product is released Details of those procedures are again not particularly relevant to the topic of the *Dynamics of Viral Infection*

Inability to produce serious illness in the vaccinated host This is of course the main criterion of attenuation Yet it should not be taken too literally An attenuated virus must multiply in the organism and a reaction of the host may be considered as a welcome sign of effectiveness of the immunizing procedure Absence of cutaneous reaction and of febrile response following primary vaccination against smallpox is regarded as immunization failure Inoculation of 17D strain of yellow fever into man is very frequently if not invariably accompanied by a febrile response at the time of viremia Lymphogenic and febrile responses in swine are concomitant with administration of attenuated hog cholera virus

An important factor in the resistance of the host to a virus is the route of administration of the infectious agent In some cases virulent viruses can produce a mild modified infection if given by an unnatural route Dr Habel mentioned viruses of fowl pox and laryngotracheitis which when attenuated are of no apparent value for immunizing purposes However, the corresponding unchanged virulent strains are excellent immunizing agents which can be administered with absolute safety provided the unnatural not respiratory route is used for inoculation

The age of the host at the time of administration of an attenuated virus may determine the intensity of its reaction For instance an attenuated strain of hog cholera may be administered to a pregnant sow and fail to elicit signs of illness in the animal However it will invariably hinder fetal development causing miscarriages stillbirths and malformation [28] The fetal tissue may be particularly vulnerable to invasion by virus particles of attenuated strains The parallel with German measles infection of man is quite obvious But just as one would not hesitate to expose children at the post fetal developmental phase to individual infection with the latter disease so vaccination with attenuated hog cholera virus should be considered as a perfectly justifiable procedure since it causes no harm to pigs from birth to death

Evidence for lack of transmissibility of the attenuated virus from an inoculated to a noninoculated host or, if transmission occurs, fixation of its modified characteristics The argument that an attenuated virus may revert to its more virulent form has been used most frequently by the opponents of live virus vaccines Since Arguments are to be avoided they are always vulgar and often convincing according to Oscar Wilde I should like to present some data which will throw additional light on the mechanism of attenuation and on the long tortuous road a viral agent has to travel before it becomes attenuated

For convenience pertinent data concerning five modified viruses are summarized in Table 4 In the case of canine distemper virus two strains

Table 4

ATTENUATION OF VIRUS VACCINES BY HOST PASSAGE

Virus	Host	Number of Passages	Attenuation for Animal Species
Canine A Distemper B	Chick Embryo	26 38	Ferret and Dog Ferret and Dog
Hog Cholera	Rabbit	> 150	Swine
Rabies	Chick Embryo	40–50 > 180	Dog Cattle
Yellow Fever	T C & Chick Embryo	0–255	Man
Newcastle Virus	Chick Embryo	I	Ch cken

A and B were adapted to developing chick embryo [1 25] The A virus was ferret adapted the other the B virus originated from a case of canine dis temper encephalitis In spite of the radically different origins of the strains the number of egg passages required to produce attenuated variants dif fered only slightly

It took 150–200 serial rabbit passages to bring about modification of hog cholera virus [26] The leporine strain at the early passage levels was as viru lent for swine as the parent porcine strain Table 5 It was decided how ever to continue the arduous labor of consecutive serial passages hoping that the virus would in the end be trained in the right direction You may observe that up to the 50th rabbit passage level a 10% suspension of in fected rabbit spleen tissue was lethal to all swine inoculated From then on a gradual process of modification seemed to take place and the strain lost its lethal properties somewhere between the 145th and 207th rabbit passage levels The results of challenge inoculation with virulent virus of swine sur viving injection of the leporine strain indicated that the modified virus re tained its antigenic capacity

It is perhaps even more instructive to trace the attenuation process of the Flury strain of rabies Originally a street virus isolated from a cadaver [27] it was passaged for 138 generations of one-day old chicks before adaptation to the developing chick embryo At the 40–50th egg passage the Flury virus was found to become non pathogenic for dogs injected parenterally and at the 180th egg passage level it became innocuous for cattle [28] Com parative pathogenic properties of the virus at the two different develop- mental stages are summarized in Table 6 It should be noted that we have here an example of almost complete cycle of attenuation although data

Table 5

EFFECT OF SERIAL RABBIT PASSAGE ON SWINE VIRULENCE

Swine Virulence Tests of Rabbit Adapted Virus		*Challenge Tests* of Surviving Swine Mortality Ratio*
Rabbit Passage	*Mortality Ratio*	
9	2/2	
11	0/2	
12	1/1	
30	6/6	
50	4/4	
80	3 5	0/2
90	2/5	0/1
97	3/7	1 4
129	2/8	0/6
135	6/25	0/17
145	1/4	0/2
207	0/11	0/11
245	0/18	0/18
284	0/12	0 12

* 1 ml of 10 000 or more LD_{50} used for challenge inoculation

discussed later will indicate that the high egg passage Flury virus is not completely free of virus particles still pathogenic for hamsters guinea pigs and rhesus monkeys

As seen again in Table 4 attenuation of yellow fever virus occurred in a gradual fashion through propagation in tissue culture and chick embryo

In marked contrast the Blacksburg strain of Newcastle virus has been found attenuated in nature [9] and is passaged in chick embryos only for the purpose of vaccine production

The analysis of the general aspects of attenuation of these viruses indicates that though other factors relative to the host and to the virus may play some role the modification occurs as the direct result of numerous passages in an unnatural host By the same token reversion of the acquired characteristics may take as many passages in the opposite direction

Of the five attenuated viruses whose history appears in Table 4 the Flury strain of rabies cannot be transmitted from host to host since it is neither excreted nor present in blood after inoculation Thus even if it multiplies in the injected animal it can only cause a self limited infection

The same can be said about canine distemper virus In numerous experiments normal ferrets a highly susceptible species were placed in contact with vaccinated dogs failed to develop any clinical signs of illness and what is more significant remained susceptible to homologous challenge [30]

The attenuated strain of yellow fever can theoretically be transmitted from host to host since it may cause viremia four to seven days after vaccination [31] Thus it may become a prey to a mosquito However millions of persons have been vaccinated against yellow fever in areas inhabited by

Table 6

COMPARATIVE PATHOGENICITY OF TWO STRAINS OF RABIES VIRUS

Virus Strain	*Egg Passage*	*Inoculation Route*	*Results of Exposure of Animal Species*					
			Hamster	*Mouse*	*G Pig*	*Rabbit*	*Dog*	*Man*
Street	0	Intraneural	++++	++++	++++	++++	++++	?
		Parenteral	++++	++	+++	+	+++	+++
Flury	40th to 50th	Intraneural	++++	++++	++++	+	±	?
		Parenteral	+	±	±	–	–	–
Flury	178th Upwards	Intraneural	–	–	–	–	–	?
		Parenteral	–	–	–	–	–	–

virus transmitting arthropod vectors,[3] and we have yet to hear about a case of yellow fever caused by reversion in properties of the 17D strain

The attenuated variant of hog cholera virus may be found in the blood of vaccinated animals However a course of five consecutive passages in susceptible pigs failed to modify the apparently fixed characteristics of the attenuated strain ■

One should also accept the fact that in rare instances because of the condition of the host attenuated virus may become involved as an associate in an actual illness A child suffering from generalized eczema exposed to contact with a pustule infected with vaccinia virus will develop eczema vaccinatum This has been known for ages but vaccinations against smallpox are carried on as usual

Once ■ virus is attenuated unexpected changes can be avoided if we deal with an homogenous virus population But even there evidence obtained with rabies virus indicates that a mixed viral population with a predominance of the attenuated forms can be successfully incorporated into a vaccine

In Figure 1 are illustrated the results of titration of the high egg passage Flury strain [8] in six species of animals all injected intracerebrally Adult mice rabbits and dogs were equally nonsusceptible to infections Rhesus

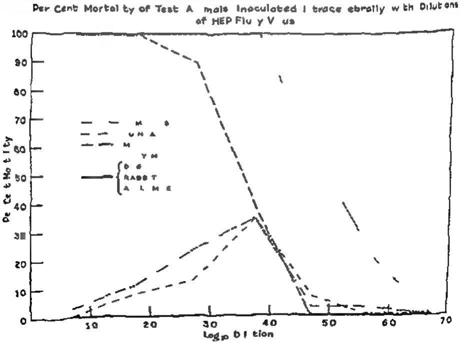

FIG 1

monkeys and baby mice remained highly susceptible to intracerebral inocu lation[28]

The results obtained in hamsters and guinea pigs are of interest since they indicate that had a concentrated suspension of infected chick embryo been used in this study both species might have been considered to repre sent nonsusceptible hosts Once the viral suspension was diluted beyond the interfering effect of particles deprived of their pathogenic properties some animals succumbed to infection These results seem to indicate that the Flury strain at this egg passage represented a mixed viral population Curi ously enough the pathogenic properties of this viral cauldron remained stable in spite of numerous efforts to change them For instance virus removed from the brain tissue of dying hamsters was still apathogenic for adult mice and when titrated in hamsters the mortality curve paralleled exactly the one shown in Figure 1 A series of ten consecutive passages through brain tissue of adult mice at different dilution levels failed also to change the pathogenic properties of the virus

While our emphasis thus far has been towards a means of control of rabies through the immunization of animals our ultimate objective is of course the application of these findings to the treatment of human beings Dr Fox[34] has employed the HEP Flury strain for the immunization of man in a clinical trial involving 90 patients Some of them received amounts of virus equivalent to 12 dog doses or 20 ml of 40% suspension No un toward reactions were observed and the safety of this immunization proce dure is beyond any doubt

I would like to remind you that the same strain is still pathogenic for rhesus monkeys injected intracerebrally and yet has no pathogenic property when injected parenterally into man This should perhaps induce us to relax judgment on characteristics of attenuated strains of poliomyelitis fit to be considered as live virus vaccines Perhaps lack of intracerebral pathogenic ity for rhesus monkeys should not be considered as *conditio sine qua non*

The problem of safety is of course a relative one Opponents of immu nization procedures with live viruses claim that these methods are unsafe in contrast to those employed in so called inactive virus preparations This is a large and I think an untenable claim I put it to you that vaccination against rabies when inactive virus preparations were used was an unsafe procedure in comparison with inoculation with living virus This claim is based on evidence If virus was properly inactivated antirabies vaccines were safe as far as their being a source of infection was concerned But killed vaccines actually presented a greater hazard than living vaccines be cause of the factor or factors producing allergic encephalitis[35] and other postvaccinal reactions Furthermore as we know immunity induced by killed vaccines is variable depending upon two things first the amount of virus present in the preparation at the time the virus was inactivated and second the manner in which the virus was inactivated It is hardly possible

to call such a method of immunization a safe procedure Opponents of live virus inoculation claim that rabies is a deadly disease and that we should take no chances even to prove the safety of the live virus immunization procedure The same argument applies to the field of poliomyelitis virus as follows poliomyelitis is a very mild disease as compared with rabies there fore the safety of a live virus vaccine can never be proven If you vaccinate 1 000 individuals the 1 001st may become paralyzed if you vaccinate 10 000 the 10 001st may become a victim of paralysis Such argument can be used *ad infinitum* in the face of the unabated zeal of the advocates of killed virus vaccines Yet immunization procedures against poliomyelitis with killed virus vaccine may be as hazardous as those using live virus If you fail to inactivate completely the living agent in your vaccine preparation the virus with which a child will be inoculated will not be of the attenuated type but of highly virulent variety The duration of immunity is undetermined at present and replicate inoculations with adjuvant preparations are inadvisable—particularly in view of our ignorance of nonspecific factor or factors present in the adjuvant preparation which may cause side reactions The hazard of using such preparations in a mass immunization program is at least as great as that of using live virus preparations However I am not going to be the one to wave a red rag in this arena to the unhappy few possessed by immunizing zeal

Evidence of adequate antigenicity following single administration of live virus preparation Not all living viruses are good antigens Of four strains of rabies virus adapted to the chick embryo and modified only one the Flury virus was found to have broad enough antigenic properties to be considered for vaccination purposes [36] Its immunizing properties remained stable through the course of 200 egg passages However constant checking of its antigenic properties is indicated particularly in view of our knowledge of the loss of immunizing properties by the 17D strain of yellow fever after numerous tissued culture passages [37 38] That a live virus vaccine may have broader antigenic properties than a killed virus preparation is indicated by the results of vaccination against hog cholera shown in Table 7 There are no known strain differences among causative agents of this disease but in 1950 a so-called variant virus[39] was discovered which seemed to infect pigs in the field vaccinated with preparations containing inactive hog cholera virus In experiments conducted by Dr C N Dale of the Bureau of Animal Industry U S Department of Agriculture results of which are shown in Table 7 it was clear that immunization with attenuated live virus vaccine (Rovac) protected animals against exposure either by contact or by inoculation with the regular virulent as well as the variant 1950 strain of hog cholera virus Although data summarized in Table 7 indicated that vaccination with crystal violet vaccine conveyed protection against challenge with the regular hog cholera virus (last column Table 7) it is also apparent that animals immunized with this inactive virus were as susceptible

Table 7

MORTALITY RATIOS OF PIGS VACCINATED WITH ROVAC UPON CHALLENGE WITH "VARIANT 1950 AND REGULAR VIRULENT HOG CHOLERA VIRUS†

Vaccine	*Type of Challenge*	*Mortality Ratios*	
		Variant 1950	*Regular Virulent*
Rovac	Injection	1 9	1/10
Rovac	Contact	1 10	1/10
CV	Contact	10/10	1/5
Cholera Immune	Contact	1/5	—
None	Contact	10/10	10/10
None	Injection	10/10	10/10

Rabbit adapted strain of hog cholera virus

† Data obtained by Dr C N Dale of B A I., U S Department of Agriculture

to infection with the variant 1950 strain as the non immunized controls (first column Table 7) These comparative figures may be considered as additional evidence for the possible broader antigenic spectrum of live virus vaccines as against those containing inactivated virus

Thus Mr Chairman in concluding may I be permitted to paraphrase a paragraph from A P Herbert s Uncommon Law and say that we have gone a long journey and met many attenuated viruses on our trip But in spite of all that was said before the behaviour of the viruses as the expert witnesses will presently testify appears to conform to no known laws whether of reason psychological or mathematical probability Their actions are impulsive capricious and incalculable Their health is delicate their nervous system easily disturbed and their moral sense negligible The merest straw is sufficient to upset their temperaments and the hopes which human beings have formed concerning them and this is especially true of those highly bred and sensitive viruses which compete professionally in the public horse races

We shall hear in the future Mr Chairman of certain viruses called favorites which because of their parentage and past performances and known ability of the scientific jockeys who will ride them are confidently expected by a majority of persons interested to defeat all their competitors in this race or that But we shall also hear that it is a comparatively rare event for the so-called favorite to finish first and in fact it has been known to finish among the last so many are the chances and accidents which in a race between different viruses may disappoint even the unanimous expectations of a people and then the general impression will remain that virology is the product of a black art administered as a mystery which none but the initiated need hope to understand

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DISCUSSION

Mechanisms of Immunity in Virus and Rickettsial Infections

DR E K WAGNER (Yale University School of Medicine) I would like to ask Dr Bodian if he thinks it might be feasible to study local tissue immunity by using excised tissues in tissue culture not merely by studying an animal that has been actively immunized to poliomyelitis virus like excising perhaps testicular tissue and testing its susceptibility to virus in tissue culture at the time of maximum antibody response

DR I M MOUNTAIN (Babies Hospital College of Physicians and Surgeons Columbia University New York New York) I have been very impressed in watching the development of the pathogenesis and immunogenesis of poliomyelitis with the close parallel with equine encephalomyelitis as first observed by E W Hurst In infection with an encephalomyelitis virus as in poliomyelitis there is a viremia which may be followed by infection of the central nervous system Another analogy is the influence of route of introduction of the virus on one hand in passive immunization to poliomyelitis as described here by Dr Bodian and on the other hand in neutralization tests with the encephalitis viruses reported in the past by Dr Olitsky and his collaborators When a neutralization test is carried out by a peripheral route such as intraperitoneal far more virus is neutralized by a given amount of antiserum than when introduced by a direct route to the central nervous system Direct routes included intracerebral as well as intranasal

These parallels suggest that for active immunization an approach effective for one may be applicable to the other infection In this regard the formalin

inactivated vaccine now effectively used against equine encephalomyelitis primarily in the horse population may point hopefully to the future of a formalin treated vaccine for prevention of poliomyelitis

DR LOOSLI I have great faith in antibodies and I am grateful for any that I have in my own circulation I think however as Dr Bodian has pointed out that local factors as well as circulating antibodies are extremely important in the pathogenesis both of poliomyelitis and influenza I would like to comment a moment or two on some of our experiments with air borne influenza virus infections in mice Mice actively immunized subcutaneously with formalin inactivated vaccines and subsequently exposed in a cloud chamber develop pulmonary infections as demonstrated by gross lesions and growth of virus in the lungs Yet these mice survive the infection and develop a marked rise in antibody titer Mice which are passively immunized before exposure to the air borne virus likewise develop a pulmonary infection and survive In contrast to the actively immunized animals no antibodies (either hemagglutinin inhibiting or neutralizing) can be demonstrated in the circulation three weeks after exposure in the cloud chamber However these animals are found to be immune to subsequent challenge with air borne viru for as long as three months This is shown by the fact that many survive challenge with airborne doses of virus equal to or greater than the first exposure Following a second challenge with the air borne virus after antibodies can no longer be demonstrated large amounts of antibody can be demonstrated in the blood Our interpretation of this phenomenon is that during the first challenge the passive antibody neutralized any virus which finds its way into the circulation so that the immune mechanism is not stimulated and thus no active antibody is produced outside the lung whereas on second challenge a viremia occurs during which the micre respond by developing a high antibody titer Our concept that a viremia is part of the pathogenesis of influenza is in agreement with that of Dr Bodian and others with respect to poliomyelitis

DR SASLAW I would like to amplify a bit more on what Dr Loosli had to say I too am grateful for antibodies but I think we frequently forget some of the cellular mechanisms Speaking about the pathogenesis of influenza and our experience with monkeys after intranasal injection we never did produce any clinical infection The only evidence we had was a leukopenia and the development of humoral antibodies However the animals that did get into trouble were those that received pyogenic organisms not at the same time but about three weeks later The only difference we could find was that their bone marrow did not respond They had an abortive leukocytosis and then came down with overwhelming septicemias With reference to the polio I would like to ask one question perhaps it has been answered in another session but how long is protection still demonstrated after this

active immunization in monkeys when challenged? What is the longest period we have had so far?

DR PAUL (Moderator) Did you direct that question to either of the two or both?

DR SASLAW Well in monkeys particularly to Dr Salk

DR HOWARD A HOWE (Johns Hopkins University) I would just like to add one or two more points in the advocacy of the chimpanzee because I think that at the present time we perhaps are being pushed to immunize people at a rate which outstrips our knowledge of immunogenesis I think that the chimpanzee is a very worthy experimental subject for studies of this kind I might say that chimpanzees become convalescents and can live in zoos from which they may be brought back and rechallenged They may be also vaccinated and challenged after two or three years they may be challenged again We know already that chimpanzees respond to inactivated vaccines with antibody levels which are comparable to those which have been discussed and we have with the chimpanzee the opportunity to study in considerable detail the events which go on in this animal on known exposure If I might anticipate answering the question that just preceded I know that convalescent chimpanzees are likely to become alimentary virus carriers after two years at about the same rate as they did after their first challenge In other words an animal convalescent and challenged immediately has about 10% chance of becoming reinfected again after two years he still has about the same chance Apropos the rather gloomy predictions of Dr Hammon this morning I think we know already that while one can limit alimentary infection with very high levels of antibody actively induced by vaccine containing inactive virus it would appear to be rather unlikely that such levels would be achieved by the vaccines which would probably be administered in practice In closing I might say that the chimpanzee does offer an opportunity to get tentative solutions to some of these long term problems which may be very slow in arriving from human populations

DR PAUL I think we might postpone the rest of discussion of these papers to the end of the afternoon but we will ask Dr Salk if he will care to answer the one question put to him

DR SALK I believe Dr Howe answered it He has had far more experience than I We have followed serum antibody levels in monkeys that have been inoculated and have found antibody in high titer after about two years we have not challenged these animals they have been saved for serological purposes For a good many months in other animals we have found resistance to challenge after vaccination I want to agree wholeheartedly

with those that have expressed views regarding the question of so-called cellular immunity At the moment a factor that influences our own investigations is that it is nice to be able to measure something If you can measure antibody and if in individuals or animals we can assume that the antibody present parallels immunity then we have a very easy and simple system both to manipulate and to serve as a guide As Dr Bodian pointed out we may well find that antibody is not the full answer merely a step and it may well be that there are more important phenomena that are active and effective in immunization

DR BODIAN I would like to add a point which I alluded to in my talk namely that although low levels of antibody in the experiments I mentioned are able to prevent paralytic infections by what seems to be purely a neural route that is by intramuscular inoculation there are exceptions which possibly depend on the virulence of the challenging strain For example the only break throughs which we had in our experiments with intramuscular challenge in animals which had received high levels of passive antibody occurred with a single strain which is known to be highly pathogenic in monkeys the old Rockefeller MV strain This fact perhaps should be viewed as a caution that conditions on which we are now focusing attention may be particular rather than general and that with a virulent strain or with one which is an antigenic variant it may take a good deal more circulating antibody perhaps more than we can produce with inactive vaccines to prevent paralytic infection There was one question directed to me about approaching the problem of local immunity in tissue culture About all I know in that field is that it is not an easy one in which to work and I would rather have somebody else assess the prospects

DR PAUL I would like to say that we have had four splendid papers and I think we have been singularly fortunate We have a few minutes for discussion and I see Dr Sabin s hand up we have been waiting to hear from him

DR SABIN Dr Habel and Dr Koprowski have just taken you on a journey introducing you to many so-called attenuated viruses I hope I may be forgiven for extending the journey a little bit and introduce you to what I might call seemingly avirulent type I type II and type III poliomyelitis viruses which we have only just recently succeeded in producing by a procedure quite different from those which have been indicated here The procedures by which attenuated viruses have been obtained before depended predominantly on introducing the viruses to an unnatural host and keeping up the serial passages with the hope that ultimately the variant virus would completely overgrow the original virulent virus This seems to have happened with the 17 D strain of yellow fever virus Both Dr Koprowski and

Dr Habel have said that it is not at all certain that it has happened with the others and one of the reasons for that may be that one has depended on complete overgrowth Now that may occur by chance but no attention has been paid to the fact that there can come a time when the avirulent particles so greatly outnumber the virulent ones that the simple terminal dilution technique might give you progeny which are avirulent This is precisely what we have done with the poliomyelitis viruses We have not used different hosts but rather we wanted to see if using the tissues of the same host we could achieve this transformation The virulence for cynomolgus monkeys was transformed by propagation of the virus in cynomolgus tissue First it was shown that virus grown in cynomolgus kidney tissue did not change in virulence when the progeny of single or small numbers of particles were repeatedly used for passage On the other hand when the multiplication was stepped up by making rapid passages with large inocula of the earliest progeny to emerge in kidney cultures we obtained what appeared to be mixtures of virulent and avirulent virus When the titrations were done with this type of mixed virus some monkeys remained perfectly well and others became paralyzed At one stage the mixtures were of such a nature that we couldn t separate the virulent from the avirulent particles by terminal dilution Finally the avirulent variety—and let me call it for the moment seemingly avirulent—overgrew the others so that with the Mahoney virus with the Leon virus and with the YSK virus we have now obtained variants which multiply to the level of 10^7 to 10^9 tissue culture doses in cynomolgus kidney tissue culture but produce neither clinical nor histologic evidence of poliomyelitis in monkeys inoculated intracerebrally with any dilution of the virus I say they are seemingly avirulent I cannot say at this moment whether the purification that has been achieved is sufficient but further work is in progress on this All that I can say is that they are highly immunogenic in that antibody is produced not only after intracerebral but also after intramuscular and after oral administration I think that some of the problems Dr Koprowski posed for us with other viruses will now have to be faced more definitely in poliomyelitis

DR O SUSSMAN (New Jersey State Department of Health) I would like to ask Dr Koprowski if in the studies that they have made whether they have attempted at all to determine what the normal infective dose is in a dog with respect to rabies That is the minimum dose that on a field trial would actually cause them to come down with rabies The question I ask is directed in that manner because I would like to know whether the safety factor which might be present in some of the vaccines which are not attenuated but are killed as Dr Koprowski indicated would not actually from the standpoint of the normal infective dose as received by a dog on the street protect to a great enough extent so that we would not have to take the hazard of going to an attenuated vaccine which may perhaps revert

DR KOPROWSKI In reply to Dr Zichis I believe I stated that the hog cholera virus was passaged five times without changing its characteristics As regards Dr Sussman I am not sure I understand the question clearly Does he mean what is the dose of fixed virus which would still infect the dog? Or does he refer to street virus? Perhaps if he will clarify his question I may be able to give the answer It is impossible to titrate street virus in dogs with uniformly lethal results In our experience we have rarely seen an experiment in which—when we injected the 10% suspension of salivary glands of dogs infected with street virus containing about one million infectious mouse intracerebral doses—100% of the dogs were brought down Therefore I could not give an accurate answer

Part IV

Laboratory Diagnosis of Virus and Rickettsial Infections

Moderator

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23

Early Diagnosis of Infections by the Psittacosis lymphogranuloma Venereum Group

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During the past two decades knowledge of the basophilic large elementary body viral agents which undergo a developmental cycle in the cytoplasm of cells derived from either entoderm or ectoderm has accrued at an increasing pace These microparasites occur naturally in frank clinical or latent infections in birds and mammals (Fig 1) Infections of avian species may be passed to man in whom they may take several clinical forms First recognized in the rarer less known more lethal forms epidemiologically attributed to exposure to parrots as early as 1879 the infective agent isolated in 1930 is now called the psittacosis virus With the discovery of other animal reservoirs and extension of the host range additional viral agents morphologically and biologically indistinguishable from the psittacosis agent have been isolated The most important exclusively human parasite of the group on account of its cosmopolitan distribution and its major public health significance [illegible] that of lymphogranuloma venereum Its relationship to the psittacosis virus was recognized between 1935 and 1942

The psittacosis agents have been held responsible principally for pneumonitis but atypical and particularly latent human infections are more common than is generally realized The portal of entry in most human infections is the respiratory tract Dust infected by dried bird droppings [illegible] frequent vehicle but infection may be contracted by handling or even by being bitten by diseased birds [illegible] [1 36] The lymphogranuloma venereum agent is transmitted venereally and the disease it produces is [illegible] disease primarily of the genital and anorectal regions but central nervous and ocular infections have occurred Other members of the group to which

Classes and Orders of Birds and Mammals in which Viruses of the Psittacosis Group Have Been Found [1]

Class Aves

Orders

Procellariiformes (1) Fulmar (L)
Ciconiiformes (1) Egret (*Egretta candissima candissima* [Gmelin])
Anseriformes (1) Domestic mallard (L)
Galliformes (2) Common fowl turkey (*Meleagris gallopavo*)
Charadriiformes (2) Willet and American herring gull
Columbiformes (3) Tame pigeon ring turtle dove and bleeding heart dove (*Galli columbia cruenta* [Gmelin])
Psittaciformes (31) Parrots parakeets and parrotlets (See *Meyer* 1948)
Passeriformes (14) Finches and sparrows
Fringillidae (7) Finches canary and titmouse

Class Mammalia

Orders

Primates Man
Ungulates Ruminants (2) Calf and sheep
Carnivores (1) Domestic cat
Rodents Muridae (2) Laboratory mouse hamster
Marsupials (2) Opossum (*Didelphis paraguayensis* and *Caluromys laniger*)

FIG 1

man is the natural host are the viruses of inclusion conjunctivitis and trachoma

The members of this growing group of morphologically similar viral agents are large enough to be seen with the ordinary oil immersion microscope when they are colored with Giemsa Castaneda or Macchiavello stains They share a developmental cycle in which colonies packed with elementary bodies arise apparently by division of larger forms which appear first in the infected cells All members of the group stimulate in the infected host several types of antibodies of considerable use in laboratory diagnosis At least two important antigens one labile and readily destroyed by heat (60 °C) the other resistant to boiling[11] or even autoclaving at 135 °C are demonstrable The labile component is destroyed by phenol hydrochloric acid or papain [4,5] The heat stabile antigen is resistant to proteolytic enzymes but is readily destroyed by potassium periodate at low concentration This behavior suggests that the heat stabile antigen is a carbohydrate [2] Antibodies to these two antigens are produced in natural infections Experimental immunization incites elaboration of additional specific toxin and infection neutralizing antibodies in readily demonstrable quantities The heat labile and heat stabile antigens can be detected by hemolytic complement fixation tests and can be differentiated

by absorption [9] By itself a positive serologic reaction is a group effect and may result from infection by any member of the group This conclusion leads directly to the problems and difficulties surrounding early diagnosis of these infections

Diagnosis of Psittacosis

Clinicians of the period 1879 to 1895 did not hesitate to describe the severe often fatal disease epidemic in households shortly after the arrival of parrots as pneumotyphus They pointed out that this illness of people living in otherwise healthy surroundings invariably coincided closely with the acquisition of sick parrots The early classical accounts of the French clinicians [8] list all the symptoms and signs observed in the most recent outbreaks From a purely clinical point of view the disease deserved the name given it by Morange[49] in 1895—psittacosis The term received its strongest support from the epidemiology and additional support from the pathology and since 1929 from the virology of this disease During the pandemic of 1929 and 1930 and the years following it became apparent that definite as is the clinical entity in man its diagnosis in any one case or in any one stage of such a case is difficult Most observers have become cautious and have realized that diagnosis must be dealt with tentatively Clinicians with extensive experience frankly state that the clinical manifestations of psittacosis are not sufficiently characteristic to enable diagnosis through them alone in an isolated case [65] Another group adopted an attitude in part prevailing today The clinical picture is not sufficiently pathognomic to allow diagnosis but given a history of association with sick birds diagnosis conditioned by experience is not difficult [20, 74] Epidemiologic and viral studies in the past 23 years have fully established the etiologic significance of a variety of birds particularly parrots parakeets and pigeons It is justifiable whenever the patient has been in contact with birds to be biased in favor of the diagnosis of psittacosis when the clinical picture points that way In fact in a recent analysis of some cases of psittacosis and pneumonia the view was expressed that in every atypical lung infection inquiries about contact with birds should be made [16, 40] Neither the physical examination nor the roentgenogram of the lung allows an unequivocal diagnosis particularly when the almost ubiquitous and early administration of chemotherapy of any acute respiratory infection may alter the clinical appearance The idea that classical psittacosis is characterized by pneumonic consolidation and a mortality rate of 20 per cent has been displaced by the knowledge that it is probably more often a mild atypical ambulatory influenza like infection Acute infectious pulmonary disease called for want of a better name atypical pneumonia unquestionably includes infection with members of the psittacosis lymphogranuloma venereum group [16, 31, 67, 68] This state of affairs demands that a diagnosis be made whenever possible if spread of the disease is to be prevented and if it is to be treated properly The attending

Classes and Orders of Birds and Mammals in which Viruses of the Psittacosis Group Have Been Found [1]

Class Aves

Orders

Procellariiformes (1) Fulmar (L)
Ciconiiformes (1) Egret (*Egretta candissima candissima* [Gmelin])
Anseriformes (1) Domestic mallard (L)
Galliformes (2) Common fowl turkey (*Meleagris gallopavo*)
Charadriiformes (2) Willet and American herring gull
Columbiformes (3) Tame pigeon ring turtle dove and bleeding heart dove (*Galli columbia cruenta* [Gmelin])
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for animal experiments by treatment with drugs[51] The mice may appear sick within 4 to 6 days and die 1 or 2 days later with lesions of seropurulent peritonitis or focal pneumonia and viremia In the exudate of diseased tissues typical elementary bodies may be readily demonstrable If the exudate is bacteriologically sterile it may be injected intracerebrally Fatal meningitis supplies impression preparations in which the developmental cycle of the viral agent can be clearly seen when stained properly It also furnishes seed for propagating the isolated viral agent in the yolk sac or amniotic cavity of the embryonated egg Experience has taught that some viral strains free from bacterial contaminants may be directly adapted from the bird host to the embryonated egg But primary enrichment of the con centration of virus in mice gives greater assurance that the agent will be isolated from human or bird sources containing relatively little virus than does immediate use of the embryonated egg When the virus is adapted to the egg reagents for preparing toxins or antigens for neutralization and serologic tests are made readily available

Whether the virus will be isolated depends largely on the availability of suitable specimens from infected human beings The outlook is not very encouraging for several reasons Between 1931 and 1944 before chemo therapy of respiratory infections became routine and indirect serologic diagnosis was limited to one or two laboratories 227 specimens of blood sputum or nasal washings derived from clinically and epidemiologically diagnosed cases of psittacosis were examined at the Hooper Foundation In only 55 (24 per cent) was the virus isolated This must in part be attributed to inadequate collection and particularly to improper preserva tion of the specimens from the patients When the test material was collected by the virologist from patients in the immediate vicinity the virus was isolated far oftener With the widespread use of chemotherapy and the gen eral dislike of the clinician to be bothered with proper collection of suitable specimens early in the acute disease the percentage of isolations dropped to a low level

Between 1945 and 1953 481 specimens from clinically and subsequently serologically proved cases of psittacosis were examined and only 23 (4 7%) yielded a viral agent As a rule the specimens were collected and shipped correctly but with few exceptions psittacosis was not suspected until after chemotherapy had been instituted Although in untreated patients the virus may be found in the blood during the first 2 weeks of an attack even in ambulatory mild infection rarely is an effort made to collect a specimen of blood aseptically before treatment is started In throat washings sputum and vomitus the virus is demonstrable up to the 26th day of the disease In a chronic case it had been isolated from the sputum in the 8th year after a severe acute attack treated only with convalescent serum[45]

Since etiologic diagnosis not subject to difficult interpretation can be made through isolation of the virus every effort must be made to combat

physician must study the patient suspected of having psittacosis meticulously —clinically roentgenologically and epidemiologically—and irrespective of a tentative diagnosis must collect a specimen of blood aseptically for etiologic diagnosis

The recent reports that an enlarged spleen is a helpful diagnostic sign clinically and pathologically deserves careful investigation [40] An incomplete survey of the clinical aspects of psittacosis lends only partial support to the contention that a palpable spleen is diagnostic The study by Sturdee and Scott[71] dealing with about 80 cases in England found only 2 cases in which the spleen was palpable One of these 2 cases was fatal The German histories suggest that the spleen may be enlarged temporarily but is not as a rule palpable or that the enlargement may be moderate and soft [2 27 28 47] Data on the gross findings in the spleen were available in 39 of 52 fatal cases of psittacosis described by Lillie [33] It was definitely enlarged in 27 cases and slightly enlarged in the remaining 12 The maximum weight was 400 g in one and was estimated at 560 g in another In 11 cases the spleen weighed over 160 g while the others not weighed all probably weighed more than that So the pathologist has recognized this manifestation but its significance as the most distinctive clinical sign deserves further appraisal Patients must be examined every day to detect this because it may be present only for relatively few days and could therefore be easily missed

Careful detailed clinical study is imperative because the laboratory procedures now available rarely permit of early diagnosis in the true sense of these words Blood specimens are almost never collected before treatment is begun so diagnosis is and in the future will be largely based on indirect serologic evidence Since the virus is so infrequently isolated during the acute disease the physician will be forced to interpret the laboratory report in the light of clinical evidence The difficulties stemming from neglect of this aspect of the diagnosis of psittacosis are eloquent in the published reports

Proof that a certain illness not necessarily a respiratory disease is caused by a strain of psittacosis or ornithosis virus rests on fulfilling the following criteria (1) The viral agent must be isolated during the acute or even the chronic phase of the disease or at autopsy (2) Complement fixing agglutinating or neutralizing antibodies should make their appearance during convalescence or more certain the titer of these antibodies should rise during recovery (3) The pathologic anatomical and experimental examination of diseased or dead birds in the patient s environment must reveal this agent The technical procedures for fulfilling criteria (1) and (3) are animal experimentation and identification of the virus

Animal Experiments Blood sputum throat washings vomitus or emulsions of organs suspected of carrying the psittacosis virus are for primary isolation injected intraperitoneally and intranasally into mice Specimens of sputum or organs contaminated with bacteria may be rendered suitable

IDENTIFICATION OF PSITTACOSIS–LYMPHOGRANULOMA VIRAL AGENTS

	P OGEN C TY				C MP EMENT F AT ON (GROU REACT N)	SUT ZAT ON OF	
	M CE SO R OUTE	S E S NT A R TO EAL	P G S I A C ER L	CEG OS R TS		TO	FECTIO
VI D S							
S C E	N C SB	o	±	+	+	C OSS	C OSS
P GEON	C	o	+	+	+	CROSS	C O S
DO E	N IC	o	+	+	+	C O S	C O S
D CK	P C	o	±	+	+	C SS	CROSS
C C E	N C	o	+	+	+	—	—
T E	C SS	++++	—	+	+	CRO	S EC F C
EG ET	C SS	++++	±	+	+	CROSS	CROSS
MAMM O G N							
M E L MO T S	N C	o	O ±	O +	+	—	S C F C
CAT	N	+++	—	o	+	S C C	S EC
O OSSUM	C	+++	o	—	+	P C C	S EC IC
C E E		+++	—	o	+	S EC C	S EC C
EW	N	+++	—	o	+	CR S	C OSS
H MAN							
SO G O IA A	P N IC SO	++++	±	+	+	R S	C OSS
O HER STR S	N C	±	± +	+	+	CRO S	C OSS
L M GR LOMA	I C	±	o	o	+	EC C	SPEC C

± S RIER W TH OCCAS ON L DEATH +++ 50 PER CE T OR MORE DEATHS
+ KI LS ++++ 100 P R CENT DEATHS

FIG 2

fatal to mice when a 20% suspension of tissue from psittacine birds turkeys or egrets is injected intraperitoneally In contrast pigeon duck and chicken strains rarely produce death of mice when introduced intraperitoneally the mice may however harbor the virus in the spleen or it may induce severe infection with extensive peritonitis and a mortality rate of 10 to 25% be tween the 8th and the 20th days

Significant are pathogenicity tests on birds All avian strains are patho genic for ricebirds Except for one mouse pneumonitis strain no mam malian strain is pathogenic for parakeets ricebirds pigeons or doves Psittacine strains are rarely fatal to pigeons on intracerebral inoculation while all pigeon and some duck and chicken strains produce fatal meningitis in pigeons The egret strain is fatal only to doves and rarely to pigeons when given intracerebrally

Such tests make valuable distinctions between strains from birds and mammals

The possibility that some mammalian strains are infectious for man is indicated by the rather frequent occurrence of complement fixing antibodies with psittacosis antigen in slaughterhouse personnel and sheepherders

(*b*) *For Toxin Action* Infected yolk sacs harvested shortly before death after draining off excess yolk and diluting in phosphate buffered gelatin solution are highly toxic on intravenous inoculation of mice weighing 10 to 12 gm In dilutions of 1 320 the induced infection may be fatal in 48 hours *Animals* inoculated with the toxin of some strains in a dilution of 1 800 may die within 5 days after having been lethargic and breathing

the prevailing idea that the complement fixation test accurately confirms the clinical diagnosis of psittacosis Physicians should be advised to collect an additional 5 ml of blood aseptically at the time they draw a sample for the customary serum tests if they have reason to suspect psittacosis The reasons for this insistent request will be apparent when the value of the complement fixation reaction is discussed later

Viral agents associated with pigeons ducks and chickens rarely produce fatal infections in mice by the intraperitoneal route so suitable preparations for microscopic confirmation must be obtained by intranasal or intracerebral injection

Many strains are of low virulence or are present in only small amounts in the blood or excretion of man Animal experiments may therefore require more than 14 days for completion Exudates derived from the pericardial or air sacs of birds dead from psittacosis or ornithosis are usually rich in viral elements They can be readily seem microscopically or they produce in mice a fatal pneumonia or meningitis within a week to 10 days

Most virus laboratories consider that the large virus elementary bodies varying in diameter from 200 to 450 millimicrons and forming basophilic cytoplasmic inclusions several microns in diameter to be a virus belonging to the psittacosis group when they react in the complement fixation test with an immune serum containing specific antibodies There may be no need for further identification and differentiation of the individual virus when the blood or sputum of the patient contains the agent isolated from birds with which he had had contact

The animal experiment has one great disadvantage It exposes the laboratory worker to the risk of infection This risk is greatly reduced when mice are used for diagnosis rather than psittacine birds as was recommended in the early days by psittacosis research workers who were not aware that these birds are frequently spontaneously infected

The simple pathogenicity test in mammals and birds may give supportive evidence that the agents from the two sources are identical If on the other hand the bird reservoir or the source of the human infection is not known (for example in pneumonitis without known bird exposure) but the sputum or autopsy lung tissue contains a psittacosis like virus clarification may have more than academic interest Until quite recently the value and dependability of the techniques of identifying and classifying members of the psittacosis group were in the realm of investigative research With recognition of the wide distribution of these agents in mammals the need for more definite differentiation became increasingly urgent Four basic tests have been developed to identify the members of the group (Fig 2) They will in time probably form a part of early diagnosis

1 PATHOGENICITY TESTS (*a*) *For Infectivity on Different Species of Animals* Usually all strains derived from birds or mammals readily infect mice when these strains are intranasally instilled Infections are regularly

and from 14 to 64 per cent of those infected with mammalian strains survived challenge. Guinea pigs surviving infections with the egret strain were completely resistant to intraperitoneal challenge with the turkey strain which induced death in control animals in 4 or 5 days. On the other hand, depending on the host origin of the mammalian strains used to immunize guinea pigs, resistance to reinfection with an avian strain has varied from 20 to 60%. These observations must be considered seriously in evaluating cross immunity tests as a basis for classifying members of the psittacosis group.

3. COMPLEMENT FIXATION TEST. Direct and indirect complement fixation tests, using yolk sac antigens prepared with avian or mammalian strains and cross titrated against antisera from birds or mammals with spontaneous infection or from guinea pigs or roosters immunized with different strains, do not distinguish individual strains. They reveal a serum reaction to the entire group and thereby furnish conclusive proof that an infecting agent belongs to this group. All strains studied have crossreacted in this test.

4. TOXIN AND VIRUS INFECTIVITY NEUTRALIZATION TESTS. In the original studies by Rake and Jones[66,5] the toxin produced by certain members of this group in the yolk sac and lethal for mice on intravenous inoculation were specifically neutralized by homologous antitoxic sera. Only homologous toxin and antitoxin mixtures of the lymphogranuloma venereum or feline or mouse pneumonitis viruses neutralized completely. Another analysis of 27 strains by the toxin neutralization test failed to indicate high strain specificity, but the strains were arranged in six distinct groups.[4] In a more detailed study of such tests at the Hooper Foundation, not only the toxin, but more specifically the viral infectivity, was strikingly neutralized. When homologous toxic virus serum mixtures in proper proportions were injected intravenously, as a rule 80 to 90% of the mice survived at least 48 hours. In the following 8 days a variable percentage, never exceeding 50%, succumbed to infection. Mice surviving 30 days usually proved to be free from latent carrier stages. The average hyperimmune rooster serum prepared to date was able to render approximately 100 000 000 elementary bodies noninfective. The protection was highly specific for certain strains, and several serotypes were identified consistently (Fig. 4). A serum prepared with a pigeon or duck strain protected against the toxin action of parakeet strains, but it specifically neutralized the viral elements of the homologous strains and those isolated from the same avian species. Exceptions were noted. (1) An antiserum prepared with a parakeet strain had a neutralization spectrum that included viruses isolated from not only parrots, pigeons and ducks, but also egrets and turkeys. (2) A serum specific for two duck viruses sometimes failed to neutralize the strain obtained from the same host of another region. (3) A feline pneumonitis antiserum prepared with a California strain specifically protected against the Baker cat strain with little or no coreaction with avian strains. (4) Equally specific was the serum prepared with the ewe abortion strains. The bovine enceph

with difficulty The ears paws and tails of those surviving 72 hours are often deep red The striking capillary dilations in the skin and in part also in the viscera are associated with hemolysis of erythrocytes and are usually observed when the highly toxic agents are tested by the intravenous route In rare instances fading of the redness of the ears heralds recovery but the mice may be found to be carriers when sacrificed 4 weeks after the infecting injection

The death curves of several strains of psittacosis virus that produce potent toxins are shown in Fig 3 The two phase curve illustrates the deaths due to intoxication within the first 48 hours of inoculation and later those due to the infective viral particles The toxin is bound to the virus particles

2 CROSS IMMUNITY TESTS Subcutaneous inoculation of mice once or several times with sublethal doses of live viral agents of this group or with large doses of killed virus and subsequently challenge with homologous or heterologous strains may disclose resistance or lack of immunity to certain strains While definite cross immunity is evident differences corresponding roughly to those reflected in pathogenicity tests become apparent A weakly virulent strain and its related types creates immunity against only homologous challenge A highly virulent psittacine or pigeon strain may induce a variable resistance against every strain thus far tested The so-called meningopneumonitis virus protects completely against the weakly pathogenic opossum virus and this in turn protects against only homologous challenge [63]

Guinea pigs surviving infections with avian or mammalian strains indicated by complement fixing antibody titers have resisted severe challenge (10−) with Louisiana (Borg)[85, 68] or egret (S E 45)[64] strains given intraperitoneally From 71 to 100% of animals infected with avian strains

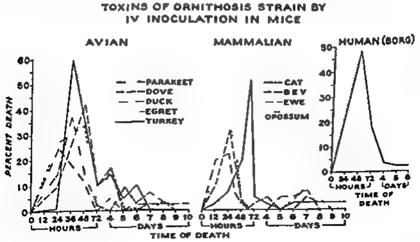

Fig 3

must be made clear Only an antigen prepared from a yolk sac infected with a known psittacosis strain should be used An antigen prepared from fertile eggs infected with the lymphogranuloma venereum virus or the commercial Lygranum must not be substituted for a specific psittacosis antigen Some evidence of this is presented in Fig 5

Among 9 sera of clinically and epidemiologically proved cases of psittacosis with isolation of the virus in one the coreaction of 5 sera in the presence of a concentrated potent commercial lymphogranuloma venereum antigen was much weaker than that with the psittacosis reagent The serologic diagnosis of 3 would have been classed as doubtful in fact in one it would have been missed entirely Even more disconcerting is the comparison of 80 sera all positive in the presence of the psittacosis antigen with both tests Exactly 50 per cent would have been reported negative had the commercial Lygranum served as the test antigen in the dilutions indicated on the vials In recent years the quality of Lygranum has been improved but the absence of coreactions continues Virus laboratories entrusted with the quantitative psittacosis complement fixation test must use potent specific reagents not Lygranum

Although the complement fixation test with cocto antigens has been widely used in the diagnosis of human avian and mammalian infections published records on systematic studies in the course of a proved psittacosis infection are quite limited The available data were collected before effective chemotherapy was known

QUANTITIVIVE COMPLEMENT FIXATION REACTIONS
SERA OF PSITTACOSIS AND LYMPHOGRANULOMA PATIENTS

NAME	PSITTACOSIS ANTIGEN	LYMPHOGRANULOMA ANTIGEN		EXPOSURE
		YOLK SAC ANTIGEN (H)	COMMERCIAL LYGRANUM (S)	
W W	1 64	0	20	GEON
J	1 128		8	PIGEON
E C	8	8	1 20	PIGEON
A H	1 64	1 4	1 40	O
U	1 8	4	20	ABO A ORY N ECTION V RUS ISOLATED
D		2	0	A A EET
	32	4	32	PARAKEET
G	256	64	80	PARAKEE
C	1 5 2	–	20	ARAKEET
D	64	2	32	LYM HOGRANU OMA
Q	1 6	8	2	MP OG ANULOMA
E R	4		0	M OGRA LOMA
	8	4	4	L M HO ANU OMA
M.C	128	8	2	LYMP OGRA LOMA
40 S RA	1 32 1 5 2	—	1 16 — 128	CO AC W TH P GEONS 5 ONTACT W H PARROTS 1 CONTACT WIT PARA E TS 2 UN NOWN 10
40 S RA	32 1 256	—	NEGA VE	C NTACT W TH EO S 17 CO TACT WI H PARR TS 2 CON ACT W ARAKE TS 2 CONTACT BI D STORE 2 UNKNOWN 7

Fig 5

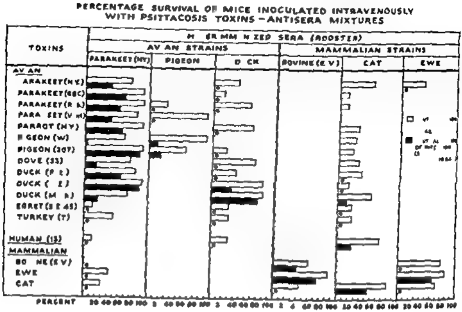

Fig 4

alomyelitis antiserum protected against infection with the ewe abortion virus as well as it did against the homologous virus The toxin and viral infectivity neutralization technique is of value in distinguishing the avian from the mammalian strains

The demonstration of specific serotypes characteristic for certain mammalian and avian strains encourages the belief that in time a few typing sera may be selected that will permit quick and ready classification of the members of the psittacosis family The presently available sera may not reflect the exact source of infection of a human pneumonitis virus when contact with diverse avian species is suspected Whenever practical recently isolated strains from human psittacosis should be subjected to neutralization tests

The Complement Fixation Test By 1930 Bedson and his colleagues[11 12] had shown that the serum in psittacosis reacts specifically in the complement fixation test when brought in contact with infectious mouse spleen suspensions Subsequent studies by this worker[8 9] definitely proved the usefulness of this test in the early diagnosis of psittacosis Preparation of stable antigens in adequate amounts offered some difficulties until cultures of the virus in Rivers L1 medium on Zinnser s solid medium[13] and subsequently antigens prepared with the yolk sac by centrifugation and boiling[65] were adopted Other antigens such as allantoic fluid from infected fertile eggs have occasionally been used but the technical procedures are now standardized to use of boiled or phenol treated yolk sac antigens One point

HUMAN PSITTACOSIS INFECTION PIGEON EXPOSURE

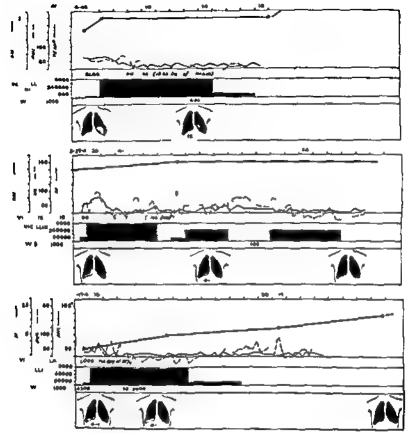

FIG 7

blood corresponded to that of the strain used in the blender In both groups an epidemiologic feature rarely emphasized was the short duration of exposure to the agent Patient M H visited a room contaminated with the virus for only an hour Patient M S remained for 5 minutes in a room where an autopsy on a pigeon was in progress As might be expected from previous experience the course of these infections varied from very mild to moderately severe Definite pulmonary infiltrations were demonstrable in the roentgenograms of 5 of the patients Despite these lesions the course of the illness was mild and atypical as was that of another patient with no roentgenologic evidence of pulmonary involvement Six patients in the two groups received penicillin in different amounts and for different periods Recovery was uneventful and complete

Pertinent to the problem of early diagnosis of psittacosis are the histories of 7 patients under constant supervision of workers fully familiar with the disease (Figs 6 7 and 8) Isolation of the virus from the blood and in [illegible] cases also from the sputum either confirmed the clinical diagnosis or proved that an indefinite illness with or without respiratory symptoms was psittacosis The source of the infection in 4 cases was unquestionably pigeons The viral agent isolated from the blood and sputum of the patients corresponded in the pathogenicity test to that obtained from squabs with which they had been in contact The viruses in these cases had the characteristics of strains derived from pigeons The source of infection of the 3 other patients was not definitely determined, but the aerosol created by a leaking Waring blender in which yolk sac material heavily infected with a parakeet strain is suspected The behavior of the virus isolated from the patients

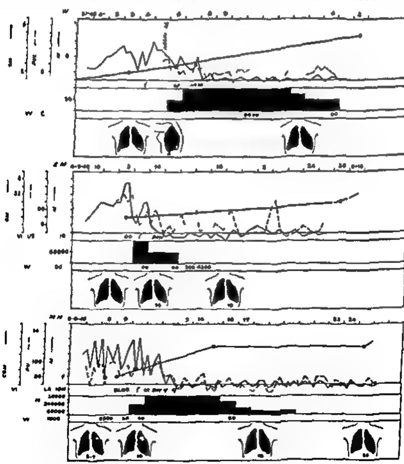

FIG 6

COMPLEMENT FIXATION ON HUMAN PAIRED SERA
PSITTACOSIS ANTIGEN

FIRST SERUM (DAY OF ILLNESS UNKNOWN)	SECOND SERUM	
C F TITER	DAY AFTER FIRST SERUM RECEIVED	C F TITER
1 8	4th	1 32
0	7th	1 256
1 2	8th	1 64
1 2	8th	1 64
1 16	8th	1 64
0	10th	1 8
0	10th	1 16
0	10th	1 64
1 8	10th	1 32
0	12th	1 2 (30th DAY 1 32)
1 2	13th	1 256
1 8	13th	1 256
1 16	15th	1 64
1 32	15th	1 64
0	16th	1 32
1 8	17th	1 256
1 32	18th	1 64
1 128	18th	1 512
1 2	20th	1 16
1 4	23 d	1 16
0	25th	1 512
0	30th	1 32
0	30th	1 128
1 32	30th	1 64
1 64	30th	1 128

Fig 9

166 specimens submitted consisted of paired sera Among these 47 in which both acute and convalescent serum was tested in 25 the titer rose significantly (Fig 9) Neither the card requesting the examination nor the subsequent letter of confirmation furnished adequate information concerning the day of illness on which the first serum specimen was collected The interval between the collection of the two specimens is known With few exceptions within 7 to 12 days the titer rose at least four to eight fold In some cases the rise appears to be more gradual If the titer had already reached 1 32 or 1 64 in the first sample the rise by the 30th day was usually only two fold In exceptional instances the rise was insignificant on the 12th day but had become diagnostic by the 30th day The data confirm previous experiences—the titers rose within 10 to 30 days of convalescence in patients subjected to specific chemotherapy and this confirmed the clinical diagnosis [44 67] Moreover in slightly over 50% of the cases the diagnosis could be made within 10 to 14 days after the onset

At the present the complement fixation test conducted with antigens of uniform potency on acute and convalescent serum is the only quick and simple means available for making an early diagnosis of psittacosis A diagnostically significant titer may not be reached until the 30th day of convalescence The antibodies may persist for weeks even years after

HUMAN PSITTACOSIS INFECTIONS

DATE	PATIENT	PROBABLE SOURCE	NCUBATION	COMPLEMENT FIXATION	VIRUS ISOLATED SOURCE AND DAY O LL	SEVERITY	DURATION
PARAKEET EXPOSURE							
3 31 45	D W	ACCIDENT W TH WAR NG BLENDER	14 DAYS	0 ---- 3rd D 1 32 ---- 0 5 BT ER TESTS	BLOOD 4 h DAY	M D (EN CILLIN)	18 DAYS
9 9 45	E M	ACC DENT WITH WARING BLENDER	NDS E NOT W LL FOR ABOUT 2 W S	8th D 4 ---- 20 D 1 6 ---- 46 D Y 1 32 ----	BLOOD 6 h D Y	VERY M D (EN C L)	17 DAYS
5 9 45	M H	ACC DENT WITH WARING BLENDER	AP ROX MATELY 7 DAYS	8th D 2 ---- 2 DAY 1 32 ---- 25 hD 1 64 ----	B OOD 8 h DAY	M D (PENICIL N)	20 D YS
PIGEON EXPOSURE							
1 27 46	M S	E POSED FOR 3 M NS IN A ROOM WHERE A TOPSY ON A PIGEON WAS IN PROG ESS	APPROX M TEL 12 19 DAYS	10th D Y 32 ---- 23rd DAY 1 128 ---- 66th DAY 1 128 ---- 95th DA 1 256 ----	BLOOD 10 h DAY SPUTUM 10 h DAY	M LD (EN C L)	14 DAYS
12 12 46	E	SAME EXPOSURE AS MS FOR LONGER PERIOD	15 DAYS	5 DAY 1 8 ---- 20th DAY 1 16 ----	B OOD 16 h DAY	NO TRE TMENT	SE ERE CO D NEU ATO Y
3 18 47	S C	TAKING CARE O SQUABS DE AR MENT O ENDOCRINOLOGY	APPROXIMATELY 10 14 DAYS	1st DAY 1 64 ---- 8th DAY 1 128 ---- 32nd DAY 1 128 ----	BLOOD 1 h DAY SPUTUM 11 h DAY	MODERATELY SE ERE (PENIC [illegible])	21 DAYS
4 11-47	P V	HOLDING AND CATCHING SQUABS R SPONS BLE FOR CASE S C	APPRO MATELY 7 DAYS	7 h D Y 2 ---- 2th DAY 1 8 ---- 9th DAY 8 ---- 3 th DAY 1 32 ----	BLOOD 7th DAY	OPERATELY SE ERE (PEN CILL N)	18 DAYS

UNLESS OTHERWISE INDICATED THE FIGURE GIVEN UNDER DURATION REPRESENTS DAYS IN THE HOSPITAL

FIG 8

Antibodies reacting in the complement fixation tests in dilutions of 1 2 to 1 8 were present in the serum of the blood specimen later proved to contain the psittacosis virus in 6 of the 7 cases The titer was 1 32 on the 8th day after onset of fever and chills in 1 case Since the day of exposure is known and 19 days elapsed between the contact and the first symptoms it seems likely that the infection progressed subclinically for several days before the onset of fever The extent of the pulmonary lesions on the 8th day of illness supports this idea The antibody titer of 1 32 reflects that infection was in progress on the 12th to 14th day A four to eight fold increase in antibody titer was invariably reached by the 20th day of illness particularly if the patients did not receive penicillin until the 5th or 6th day of their illness There is some evidence that early treatment depresses antibody formation but the sluggish rise in titer is probably not entirely attributable to treat ment The same trend has been observed in untreated patients [44] The level of antibodies usually reached its highest point around the 30th day

Unfortunately systematic studies on patients treated with aureomycin or terramycin are not yet available One must depend on observations on patients considered to have psittacosis because they had been exposed to sick parakeets or infected turkeys and because they manifested clinical evi dence of the disease No effort was made to isolate the virus Most of the patients had been treated with aureomycin The physicians who submitted these specimens influenced by reports on the increased frequency of psit tacosis due to the wide distribution of imported parakeets requested labora tory assistance during the early months of 1953 Only 47 (35%) of the

fusing because over 40% of the sera gave positive Wassermann and Kahn reactions Although the complement fixation test with Lygranum may provide a lower percentage of reactors than the psittacosis antigen there is no certainty that the titers do not merely represent group reactions [54]

Recently an entirely new aspect of this test came to light The accidental discovery of pulmonary brucellosis prompted adoption of unorthodox methods of investigation at the Hooper Foundation Instead of accepting the request of the physician that the serum be tested for antibodies against the disease noted on the accompanying cards tests with a battery of antigens are run The first startling observation was in a case of febrile pneumonitis indicated by cough and blood streaked sputum in a young Negro employed as a slaughterer The tentative diagnosis was psittacosis since the complement fixation reaction in the presence of the psittacosis antigen was positive in a dilution of 1 64 with the lymphogranuloma venereum antigen it was 1 16 and the Wassermann and Kahn reactions were negative When the titer in the agglutination test with two brucella strains was 1 320 diagnosis was pursued further Sputum specimens were tested intranasally on mice *Brucella suis* not the psittacosis virus was isolated from the lungs of the infected mice The correct diagnosis was brucellosis with pulmonary complications The positive complement fixation reaction with the psittacosis antigen was considered anamnestic of a previous probably latent infection with a member of the psittacosis group possibly of mammalian origin

In another case the original clinical diagnosis of psittacosis had to be changed to brucellosis because the psittacosis complement fixation reaction was negative and brucella agglutinins and complement fixing antibodies rose steadily

Five patients with clinical diagnoses of leptospirosis because they were suffering from icterus and hepatic disease and two had enlarged spleens had no agglutinins for the leptospira usually encountered in the United States All had however either a high or rising psittacosis complement fixation titer On inquiry it was learned that 2 had had definite contact with birds and both infections responded to penicillin therapy Another had had lymphogranuloma venereum and 2 others gave on further questioning a history of liver disease Recent recognition of the prevalence of leptospirosis has evidently focused the physician s diagnostic thinking on this infection He must be careful to avoid being misled by the present popularity of leptospirosis

It is disconcerting that these observations support the repeatedly expressed view that psittacosis may masquerade under a diversity of clinical symptoms and only careful laboratory studies may furnish an accurate etiologic diagnosis

Finally on several occasions a diagnosis of Q fever has had to be corrected in favor of psittacosis because of a rise in specific antibodies and an

untreated psittacosis [44] A few observations indicate that after chemotherapy the persistence of the antibodies over long periods is less frequent The history of a chronic carrier of the psittacosis virus in the respiratory tract with a complement fixation titer of 1 256 for 8 years [4] supports the view that the complement fixation reaction may be a true indication of the presence of the virus in the host tissues and might be useful in the evaluation of cure of the infection

There should be no need to emphasize that a single positive complement fixation reaction is not diagnostic Agents capable of eliciting such antibodies are ubiquitous and these antibodies may persist In reported histories of suspected cases of psittacosis the diagnosis is sometimes carelessly dismissed with the information that the complement fixation test was positive for the psittacosis lymphogranuloma venereum group Too often neither the titer of the reaction nor the day on which the serum sample was drawn is mentioned Too often only one report is given Physicians must appreciate that a positive serologic reaction is a group reaction and that titers of 1 16 or higher may result from latent infection with any member of the group A good example is set in the report of a series of cases of atypical pneumonia in West London a titer of 1 160 or lower was ignored if there was no other evidence of psittacosis [16] The laboratory can do no more than report the complement fixation titer and leave the interpretation to the physician At best a single complement fixation reaction in a titer of 1 64 or above is suggestive only that the patient is or has been infected with a member of this group but that the illness at hand is not necessarily psittacosis

The complement fixation reaction may be used for surveys if its limitations are appreciated In an at random sample of 455 sera from persons not clinically infected with any member of the group 10% had a titer of 1 8 5% of 1 16 1 5% of 1 32 and 1% of 1 64 There was no significant difference between the titers of a group of about 100 inmates of an asylum who had had no opportunity for contact with birds for at least 5 years These observations were made in Holland where lymphogranuloma venereum is very rare [17 18 19] Similar findings have been reported from other countries In Denmark for example of 132 sera from 116 patients received for cold agglutination test presumably because of atypical pulmonary disease 5% had a titer of 1 15 3% of 1 30 4% of 1 120 3% of 1 480 and 1% of over 1 960 [6] Sera of caretakers in zoological gardens or owners of aviaries or infected pigeon flocks may yield titers of 1 16 or even 1 32 without any history of infection [43]

Occasionally the magnitude of the titer may be correlated with the length or intensity of exposure to infected birds For example some workers who had eviscerated pigeons for over a year have had stationary titers of 1 64 and above Of limited significance are serum surveys of Negroes working on poultry farms The results and their interpretation were particularly con

cordance in 13 patients[13] More recently by removing the group antigen through chemical treatment with potassium periodate or dilute acids preparations have been obtained that elicit positive specific or largely specific allergic reactions[3,4,5] In fact in patients with lymphogranuloma venereum only the homologous extract elicited a definite response In 1 case of psittacosis the use of an acid extract was equally specific Thus there is good evidence that acid extracts give specific reactions when injected intradermally to human beings infected with viruses of this group

During the past few years however three reports by British clinicians lend little support to this hope Of 3 patients with psittacosis only 1 had a positive intradermal reaction with the Frei antigen on the 42nd day of convalescence[1,3] Three specific antigens were tested on 2 patients with proved psittacosis due to contact with a sick parakeet one gave a positive intradermal reaction only with the ornithosis antigen on the 30th but not on the 35th day of his illness The test with the Frei antigen was negative in this patient but it was positive in the second patient when tested on the 54th day of convalescence[1] For the present the reported facts can be regarded as interesting perhaps suggestive but since the nature or origin of the ornithosis antigen is unknown one cannot draw any further conclusions Certainly many more tests with specific antigens must be made before an opinion concerning skin sensitivity as a diagnostic sign in psittacosis can be formulated It is evident now that (1) the intradermal test is not suitable for early diagnosis and (2) sensitivity to presently available antigens occurs far too irregularly to be dependable While skin sensitivity as a rule develops in patients with lymphogranuloma venereum the more acute nature of psittacosis which may be rapidly terminated by modern chemotherapy may be responsible for the irregular appearance or lack of allergy Until the properties of the specific skin reactive antigen particularly its relation to the specific toxic factor of the different members of the psittacosis lymphogranuloma venereum group are defined the skin test remains as a subject for further investigative research

Diagnosis of Lymphogranuloma Venereum

The literature on the clinical aspects of lymphogranuloma venereum and on the exact value of the different laboratory diagnostic procedures is already quite considerable It would be presumptuous to attempt detailed analysis The clinical course of this infection rarely calls for an early diagnosis The presentation that follows is concerned with the laboratory investigations to aid the clinician in diagnosing infections with this virus about which he is in doubt

Microscopic Examination and Animal or Egg Inoculation

Microscopic examination of bubo pus may prove useful but as a rule it is difficult to recognize the elementary bodies with any degree of certainty

absence of antibodies for Q fever It is recommended that other virus laboratories follow the procedures outlined and make available their findings in order that the serologic diagnosis of psittacosis may be better understood

Search for More Specific Diagnosis The serologic specificity of the direct and indirect complement fixation conglutinating complement absorption hemagglutination inhibition and neutralization tests has been explored Group specificity has invariably prevailed and the few minor antigenic differences have rarely been great or constant enough to be of practical value [9,70] Limited progress has been made by removing the group antibodies by absorption with the group antigens If a complement fixation test is made with such an absorbed serum and fresh unheated lymphogranuloma venereum virus as antigen instead of the usual boiled antigen the reaction becomes specific Cross absorption tests have shown that the labile antigen in the lymphogranuloma venereum agent the virus of enzootic abortion meningopneumonitis and other members of the psittacosis group are specific [13,[illegible],66] Whatever combination of serum and heated virus may be used for absorption the antibody only to the unheated homologous suspension is left

For the study of unidentified viruses the value of sera absorbed with heated virus is unquestionable but it is hardly suitable for routine use The amount of specific antigen in freshly made suspensions of unheated active *virus is unpredictable Routine virus laboratories should not be encouraged* to use live virus as antigen The risk is too great [7,73]

A more promising technique of securing specific antibody reactions has not been explored as vigorously as limited observations warrant [6,7,33,37] Formalin killed purified and concentrated suspensions of elementary bodies prepared from allantoic fluids or even yolk sac material are specifically agglutinated by sera of chickens injected with unheated viral material It would apparently be rewarding to investigate the specificity of the agglutination reaction Production of potent antigens in amounts adequate to meet the requirements for daily routine use might at first offer some problems The complement fixation test with boiled antigen might serve as a screen the investigator bearing in mind that the group specific reactions must be interpreted with other findings

Skin Sensitivity Tests Since it was demonstrated that the heat stabile antigen of the psittacosis agent provokes allergic reactions in sensitized guinea pigs and rabbits [93,60] few observations on intradermal tests on patients have been reported The Frei test with the yolk sac antigen may be positive in patients with psittacosis [58] and the psittacosis antigen prepared in a similar manner has given strong reactions in patients with lymphogranuloma venereum [13,55] Parallel intradermal tests with steamed lymphogranuloma venereum and psittacosis antigens on patients with lymphogranuloma venereum furnished as might be expected since the effective antigen in both would be the group antigen considerable con

Complement Fixation Test During the past 15 years attempts have been made with equivocal results to demonstrate complement fixing antibodies in sera of patients with lymphogranuloma venereum using antigens varying in potency according to the amount of virus present With the introduction of yolk sac antigens extensive serologic investigations were carried out [13 8 59 68] The criteria established for the serologic diagnosis of an infection with any member of the psittacosis lymphogranuloma venereum group namely a significant rise in antibody titer can rarely be met Most patients with this disease are first seen in the secondary stage of the infection when inguinal adenitis draws attention to the infection and by that time antibody formation is usually well advanced Other patients particularly women become aware of the infection only when it has reached the tertiary stage and usually rectal lesions draw attention to its presence Only rarely is it seen early enough so that formation of antibodies can be followed The investigator therefore relies on a single observation As would be expected nonspecific reactions have occurred in patients with upper respiratory tract infections [0 4] A purely serodiagnosis of lymphogranuloma venereum by means of a single test with Lygranum is not possible [4] Unless there are clinical manifestations a titer of 1 32 merely indicates recent infection by some member of the psittacosis group

Recent extensive and detailed independent investigations with standardized antigenic procedures by many workers[15 20 34 76 77] justify the conclusion that in a patient with symptoms of lymphogranuloma venereum a titer of 1 32 or 1 40 or higher is beyond the range of nonspecific effect and indicates active infection The complement fixation test is more specific than the intradermal test Furthermore the published data indicate that complement fixing antibodies develop before the Frei test becomes positive but the latter test may remain so for many years Both tests must be carried out in cases of suspected lymphogranuloma venereum because the serologic effects of intensive therapy are not known The titers of repeated samples may cover a wide range and it is very difficult to assess how far this may be due to intensive therapy Observations on experimental infections clearly show that if the treatment eliminates the virus the fall in titer is strikingly discernible [76] The complement fixation test is useful not only in diagnosis but may also prove of value in determining the effect of treatment on the infection

Attempts of Bedson Barwell and their associates to devise a more specific test by absorbing the group antigen from the sera and to use live unheated antigens have already been described and the disadvantages have been discussed It is appropriate to repeat again and again that in its present form the complement fixation test for lymphogranuloma venereum with the heat stable antigen cannot with any degree of confidence be used to screen population groups to gauge the incidence of this infection The greater incidence of reactions to a titer of 1 40 or higher may indicate latent infection in the Negro population [39] but the fairly large number of positive reactions in pa

unless they are numerous Microscopic study of biopsy material from the granulomatous lesions furnishes worthwhile information as the description of the histopathologic changes by Smith and Custer[69] indicate Stellate abscesses and suppurative granulomas are characteristic of the disease but not pathognomonic Microscopy then is of limited use

Attempts to transmit this infection to animals or embryonated eggs in the early stages of the disease are definitely indicated The methods have been fully described by Wall[75] and Beeson and his associates [14] Isolation and identification of the viral agent is of course unequivocal proof but the procedures are time consuming and without adequate experience are not readily achieved

Consequently the laboratory must rely largely on indirect methods The Frei test and the complement fixation test are easy to make and give an answer in a relatively short time

Skin Sensitivity Test The Frei test [5] is based on the skin sensitivity provoked by intradermal injection of inactive virus It can be made with bubo pus obtained from human lesions infected mouse brain or infected yolk sacs Infected yolk sacs are now used as standardized antigens almost exclusively The virus is inactivated by heat and a control made from normal yolk sacs is used to detect the rare egg sensitive individual The test and control materials are injected intradermally at separate sites in the forearm in a dose of 0 1 *ml and readings are taken at 48 and 72 hours* Erythema regardless of its extent surrounding the point of injection is of no significance A positive reaction is central induration with surrounding edema when the control reaction is slight induration of an area less than 5 mm in diameter The erythematous crown of a positive reaction is not always readily perceived in the Negro

Use of this test has helped in the recognition of lymphogranuloma venereum in its multiple manifestations and has removed some differential diagnostic difficulties The reaction is generally considered to be highly specific It can be elicited after clinical recovery and may at times be life long A positive reaction therefore does not necessarily mean that the present illness is being provoked by this virus Nor does one negative reaction rule out lymphogranuloma venereum The significance of this test is similar to that of the Mantoux test in tuberculosis Some positive Frei reactions might result from previous infection with other members of the psittacosis group but the evidence thus far published is too meager to permit definite conclusion [81] *In doubtful cases it may become necessary to institute* tests with the acid extract of the lymphogranuloma venereum virus which according to Barwell [84] gives specific positive reactions More extensive trials are warranted to assess the value of this test In conjunction with the complement fixation test a positive Frei test has definite value The results of the two may disagree and in this instance the complement fixation test is the better guide [0 41]

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tients with no venereal disease in a country as free of venereal disease as Denmark requires another epidemiologic interpretation In the future efforts should not be spared to supplement serologic tests with intradermal tests carried out with extracts of several psittacosis and lymphogranuloma viruses treated with dilute hydrochloric acid But there is no assurance that this combination will prove informative since the Frei test with heated antigen may be less correlated with the titer of the complement fixation test in latent than in clinical infections

Diagnosis of Inclusion Conjunctivitis and Trachoma

These two viruses may well be considered together because the laboratory diagnosis in both consists in finding cytoplasmic inclusions in epithelial cells Clinical evidence invariably assists the ultimate diagnosis The distinction between the virus of inclusion conjunctivitis and the virus of trachoma rests on the dissimilarity of the clinical pictures Trachoma alone gives rise to cicatricial changes and panus Although trachoma has been experimentally transmitted to the vervet monkey and *Macacus rhesus* such tests are too expensive and too time consuming for routine use The virus of inclusion conjunctivitis has not been cultivated in eggs or animals

The observation that sera from patients with trachoma may fix complement with psittacosis and lymphogranuloma venereum antigen[60] is in the light of more extensive studies less significant than it was considered to be at the time it was made

Through the courtesy of Prof Yukihiko Mitsui Kumamato University Medical School in Japan sera of 17 patients with trachoma were made available for quantitative complement fixation tests with heated psittacosis and lymphogranuloma antigens The only significant reactions were in the serum in an acute case The titer with psittacosis antigen was 1 16 during the first 4 weeks of the illness

In another series of 199 sera secured through the Chief of the Division Medical Section of the Far East Command the serum reactions of treated and untreated trachoma patients were compared with those of persons living in the same area The distribution of the complement fixation reactions on a percentage basis for each serum dilution is given in Fig 10 The percentage of strongly positive reactions was much larger in the group of persons reported as free from eye disease The distribution is quite similar to that reported for other population groups surveyed for the prevalence of lymphogranuloma venereum The significance of the percentage of reactions in the group of trachoma patients is obviously marred by the general high incidence of infections due to other agents of the psittacosis lymphogranuloma venereum group which apparently prevails among the people of the Japanese islands Whether it is latent lymphogranuloma venereum or psittacosis could not be determined The rather ubiquitous prevalence of parakeets may be just as important as the venereal disease

PERCENT REACTORS OF SERA FROM TRACHOMA PATIENTS AND THE POPULATION AT LARGE TESTED BY COMPLEMENT FIXATION

SERA TESTED	ANTIGENS	SERUM DILUTIONS								
		0	1 2	1 4	1 8	1 16	1 32	1 64	1 128	1 256
92 TRACHOMA PATIENTS	PSITTACOSIS	206	173	217	161	195	20	0	10	0
	LYGRANUM	293	163	239	195	76	31	0	0	0
107 POPULATION AT LARGE	PSITTACOSIS	81	102	140	196	251	140	56	18	18
	LYGRANUM	112	75	195	233	224	112	37	09	0

FIG 10

These studies do not necessarily invalidate the conclusions that complement fixation tests on trachoma patients show an antigenic relationship of this virus to the psittacosis lymphogranuloma venereum group They do re emphasize the fact that correct interpretation of one serologic finding without epidemiologic histories is most difficult It must be carefully weighed before far reaching deductions regarding relationships are drawn It is obvious that the complement fixation reaction is not a specific test for trachoma or inclusion conjunctivitis A serologic test is not available at present In the few cases of trachoma in which the Frei test has been used it has invariably been negative Skin tests with inactivated trachoma material have given somewhat inconclusive results [15] The early diagnosis is consequently confined to the microscopic examination of suitable Giemsa or Castaneda stained smears made from conjunctival scrapings

Summary

The nature of the disease and of the presently available laboratory tests make early diagnosis of psittacosis and ornithosis tentative and dependent on clinical and epidemiologic observations The clinical picture ranges from mild respiratory infection to fatal atypical pneumonia Normal respiratory rate relatively slow pulse normal leukocyte count absence of classical signs of pneumonia non productive cough and lung consolidation revealed by x ray are characteristic but not pathognomonic Physicians faced with a patient with symptoms and signs of psittacosis or ornithosis should take a blood sample adequate to provide material for isolation of the virus and for the complement fixation test before treatment is begun If antibiotics are given before the samples are taken efforts to isolate the virus are likely to be futile No effort should be spared to obtain a specimen of sputum however small even if expectorants must be used Whatever material—blood sputum pleural fluid vomitus or throat garglings—must be promptly frozen in a sealed container and sent to a laboratory capable of diagnosing the in

fection Additional samples (each to yield at least 4 or 5 ml of serum) should be collected on the 8th 16th 30th and 40th days so that the antibody pattern can be observed To be at all conclusive the titer should rise after about the 14th day of illness to at least 1 32 If the serologic response follows the typical pattern—weak during the acute phase strong and persistent through convalescence and declining slowly thereafter—the patient probably but not beyond doubt is infected by a member of the psittacosis group The laboratory should eliminate bacterial contaminants from the specimen probably with antibiotics inject a suspension into mice intraperitoneally and intranasally and observe the results In the complement fixation test stable antigen prepared with a known strain of psittacosis not lymphogranuloma venereum virus should be used If possible the laboratory should study the virus further with pathogenicity cross immunity and toxin and infection neutralization tests

Probably only in surveys is lymphogranuloma venereum ever diagnosed early in the infection The symptoms and signs characteristic of the secondary stage usually send the patient to the physician He should be led by these to make the Frei intracutaneous test and to send serum to a qualified laboratory for a complement fixation test Diagnosis by culture and histopathologic study may also be attempted A positive Frei reaction consists in central induration surrounded by edema at the site of injection of a standardized lymphogranuloma venereum antigen and slight induration of an area less than 5 mm in diameter at the site of injection of normal yolk sac (to detect the egg sensitive individual) The results should be observed 48 and 72 hours after injection The allergic state indicated by a positive result may persist for years and should therefore not be depended on too heavily as a guide to the present illness Since the infection is old by the time it becomes clinical the complement fixation titer is high 1 32++++ or more

The possibility of cross reactions of viruses of the psittacosis lymphogranuloma venereum group in the complement fixation test should be borne in mind in considering the results It should go without saying that careful history taking contributes valuable supportive diagnostic information

Papillary follicular hypertrophy of the conjunctiva and abundant conjunctival exudate containing numerous neutrophils involving the cornea is characteristic of trachoma acute or chronic Upper portions of the conjunctiva and cornea are more involved than the lower The reverse is true in inclusion blennorrhea In most instances trachoma can be diagnosed through clinical features alone If necessary epithelial scrapings can be submitted to the laboratory for morphologic study and follicular expressions for cytologic study In inclusion blennorrhea material is frequently obtainable only by removing the entire follicle cellular elements shown in the smears are predominantly lymphocytes and a few small macrophages with little debris

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24

Early Diagnosis of Smallpox

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Under this title will be discussed the early diagnosis of variola major and minor (alastrim) vaccinia and cow pox with only slight reference to varicella (chicken pox) It is not intended to give an historical account of the development of the various tests but a few references will be made to *comparatively recent papers from which further information may be ob* tained

The tests and techniques described have all been in use for some years in other laboratories as well as our own and their reliability and repro ducibility has been demonstrated Various modifications can obviously be made to suit local circumstances and the suitability of doing certain tests and the interpretation of the results of others may vary from one place to another

The Central Public Health Laboratory at Colindale London receives material for smallpox diagnosis from anywhere in England and Wales except Liverpool and the adjacent areas for which Professor Downie is responsible We also accept material from Malta and Gibraltar and any other place out side England which requests our help Vaccination has not been compulsory in Great Britain since July 1948 so that the early diagnosis in the labora tory has become of increasing value and we have taken additional steps to prevent the disease arriving under the guise of chicken pox by encourag ing the sending of specimens from persons with suspicious rashes en route by ship from certain areas where smallpox is epidemic or endemic We cannot of course contend with introduction by air travellers in the same way but encouragement of physicians and other health officers to use the laboratory may help to neutralize this danger It is possible that similar methods may be of value in other countries and in the general control of the spread of smallpox

There are three main types of tests available each of which has its special value depending upon the stage of the disease and the type and quantity of material that can be obtained from the patient

1 The microscopic examination of smears made from scrapings of the base of lesions on clean glass slides and suitably stained for the presence of virus elementary bodies This may provide an answer within one hour of the receipt of the specimens at the laboratory

2 Complement fixation tests which may be applied in one or other of two ways at any stage of the disease from the onset of fever to the second month of convalescence i e (a) to detect the presence of antigen in either the blood in the first few days or in the skin lesions from the time of their appearance until their disappearance or (b) to detect antibody in the patient s serum A result is available in 24 hours by either of these tests

3 Virus culture may be attempted on the chorioallantoic membrane of fertile fowls eggs with blood in the first two or three days of illness or material from skin lesions from the time of their appearance to their dis appearance This is the most sensitive test but a result will not be available for at least 48 hours and usually 72 hours

Table 1

MATERIAL TO BE COLLECTED AT DIFFERENT STAGES

Material	*Pre eruptive*	*Maculo Papular*	*Vesicular*	*Pustular*	*Scabs*
Clotted Blood (Serum & Cells)	X (v & a)	X (v & a)	X (v)	X (ab)	X (ab)
Smears	0	X (m v & a)	X (m & v)	X (v)	0
Vesicle Fluid		0	X (v & a)	0	0
Pus	■	0	0	X	0
Tops of Lesions and Scabs	0	■	X (a)	X (a)	X (v & a)

m = microscopy ab = antibody

v = virus X = available

a = antigen ■ = specimen not available

Table 1 shows the type of specimen that it will be useful to examine at each stage and the test for which it will be used The equipment that should be available when one is preparing to collect material from a patient with suspected pox infection consists of

1 A sterile venipuncture needle with or without a syringe and a sterile bottle for collecting 10 cc sterile blood

2 A Hagedorn needle in a tube or a scalpel for scraping lesions and making smears

3 4 to 6 clean glass microscope slides in a box

4 2 to 3 capillaries in a screw-capped bottle If possible a small rubber

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The tests and techniques described have all been in use for some years in other laboratories as well as our own and their reliability and reproductibility has been demonstrated Various modifications can obviously be made to suit local circumstances and the suitability of doing certain tests and the interpretation of the results of others may vary from one place to another

The Central Public Health Laboratory at Colindale London receives material for smallpox diagnosis from anywhere in England and Wales except Liverpool and the adjacent areas for which Professor Downie is responsible We also accept material from Malta and Gibraltar and any other place outside England which requests our help Vaccination has not been compulsory in Great Britain since July 1948 so that the early diagnosis in the laboratory has become of increasing value and we have taken additional steps to prevent the disease arriving under the guise of chicken pox by encouraging the sending of specimens from persons with suspicious rashes en route by ship from certain areas where smallpox is epidemic or endemic We cannot of course contend with introduction by air travellers in the same way but encouragement of physicians and other health officers to use the laboratory may help to neutralize this danger It is possible that similar methods may be of value in other countries and in the general control of the spread of smallpox

Technique of Tests

Microscopic Examination of Smears The smears are stained with Paschen s or Gutstein s or similar suitable stains In a clean and successful preparation numerous typical elementary bodies can be seen (Fig 1) In varicella zoster or herpes simplex only a small number of elementary bodies are usually found The value of the microscopic examination is very much dependent upon the cleanliness of the slide and the experience of the person making the smear and staining it A positive report is always pro visional and a negative report for virus is of little significance However

FIG 1 Smear from base of smallpox vesicle showing remains of cell and numerous elementary bodies Stained by Gutstein s alkaline methyl violet method × 1400

multi nucleated giant cells are frequently found in smears from lesions in herpes and varicella but not in variola as originally pointed out by Tyzzer [5] a fact which may help in giving a presumptive negative report

Complement Fixation Test This may be applied in one manner or an other at any stage from the onset of fever to convalescence As already stated essentially the same technique may be performed to detect either antigen or antibody The commonest application of the test is for the de tection of antigen in the presence of antivaccinia rabbit serum The ma terials which may be examined in this way are serum scrapings of macules or *papules vesicle fluid pustule fluid tops of vesicles or pustules* and scabs crusts or seeds The second type of test is of course the use of a variola antigen from man or a vaccinia antigen from rabbits to detect anti body in the serum at varying stages of illness in patients who have not been vaccinated for six to nine months or more (or to detect a rise in titre in two

teat should be included to aid in the collection of vesicle fluid or pus An alternative is a Pasteur pipette with teat enclosed in a tube

5 A small screw capped bottle for tops of lesions or scabs which may be obtained by using either the needle or scalpel but a pair of fine forceps may also be useful for this purpose

Specimens should be encased in a metal container for transport to the laboratory The form accompanying the specimens should state the date of *the onset of illness when the patient was last vaccinated and whether there* is any past history of chicken pox

The specimens need not be transported packed in ice unless the atmospheric temperature is above 37° C and more than 24 hours delay is expected before they reach the laboratory The tests for antigen will not be affected by the higher temperatures but small amounts of virus might be inactivated and give negative results on egg culture The material needed is detailed below

Collection of Specimens

Blood Collect at least 5 ml of venous blood in the clean dry sterile screw capped bottle or tube with rubber stopper The blood may be frozen after the serum has been separated from the clot Both serum and clot should be sent to the laboratory

Smears Clean the surface of the lesions with spirit or alcohol In the macular or papular stage the superficial epidermis is removed and smears are then made from scrapings of several lesions onto clean glass slides The slides are allowed to dry in the air and are *not* heated If more than one smear is made the slides should be placed face to face and separated by adhesive tape or match sticks and the surfaces on which the smears are made should be clearly marked In the vesicular stage the tops of lesions are cleaned opened the fluid collected and the tops removed and then *the bases of several lesions are scraped to make the smears In the pustular* stage smears are of little value for microscopy but are a convenient method of collecting pus for antigen or virus culture tests

Vesicle or Pustule Fluid Clean the tops of lesions open lesions and collect the fluid in capillaries with open ends The fluid should run in by capillary attraction but if difficulty is encountered help can be obtained by the use of a small capillary teat or fluid can be removed by the use of a Pasteur pipette If capillaries are used only the end distal from the specimen needs to be sealed and the capillaries are placed in a sterile bottle or tube with rubber stopper

Tops of Lesions or Scabs Clean the tops of lesions with spirit or alcohol or collect the crusts etc from six or more lesions into a sterile screw capped bottle or tube with rubber stopper

of 9% NACL is added mixed with the powdered crust and allowed to extract at 4 C for 1½ to 2 hours (The 9% NACL is considered to help in the disintegration of the cells and liberation of antigen) After extraction distilled water is added to ten times the volume of the salt solution making a 1/100 dilution of the crusts in normal saline The suspension is then transferred to a centrifuge tube or bottle for centrifugation at 2000 r p m for ten minutes The supernatant fluid is removed heated at 58 C–60 C for 30 minutes and used as antigen As previously stated the minimal volume of antigen required for the test is 0 6 ml so that if the original material weighs less than 0 01 g it should be taken at this figure and made up accordingly In the latter case a negative result is of doubtful significance If only a single scab is received a small piece must first be removed to make a separate suspension in broth containing antibiotics for egg culture

Complement Fixation Test The haemolytic system consists of 2 5% sensitized sheep cells and 3 M H D complement An overnight fixation at 4 C is preferable However if a large amount of antigen is available a shorter fixation of 4 hours at 37 C can be used and if positive may be accepted but if negative an overnight fixation should also be done

When testing an unknown antigen doubling dilutions of the test antigen and the known positive antigen are made in duplicate the unknown antigen starting undiluted To one of each row of antigen dilutions is then added 1 volume (0 1 ml) of normal rabbit serum and to the other two a volume of hyper immune rabbit serum The dilution of the immune serum used is governed by that which gives an optimal titre with the positive control antigen One volume (0 1 ml) of 3 M H D of complement is finally added The tubes are shaken and the racks put at 4 C overnight for fixation Next morning the racks and sensitized cells are removed from the cold to the bench and left for 30 minutes to warm up before adding 2 volumes (0 2 ml) of the sensitized cell suspension to each tube The tubes are then shaken placed in a water bath at 37 C for 30 minutes removed and left on the bench to settle for one hour and read unless completely negative on removal from the bath

A varicella antigen should always be included as a negative control The majority of specimens sent for test come from varicella patients but we make no attempt at present to obtain a positive varicella test because the material from chicken pox particularly crusts provides a very poor antigen quantitatively and human varicella convalescent serum apparently contains relatively small amounts of complement fixing antibody The heat stable complement fixing antigens produced by the above methods appear to be similar for the three pox viruses so that this test will only differentiate them from varicella or herpes or other agents in which the test will be negative but of course this is the most important function of the test If the clinical or epidemiological investigations do not permit an immediate diagnosis within the pox group then one must wait for final differentiation by the

samples of serum from patients vaccinated less than six months previously who may come under suspicion very very rarely) The materials to be examined in either test are put up with previously determined optimal doses of positive serum or antigen respectively Usually 0 1 ml volumes are used The material on test is diluted to provide sufficient quantity to carry out the test or tests the results of which may be capable of interpretation Over night fixation (16 to 18 hours) at 4° C gives the best results Positive serum is obtained from rabbits hyper immunised with rabbit adapted vaccinia

Preparation of Antigens FROM BLOOD IN THE ACUTE STAGE UP TO THE FIFTH DAY OF DISEASE The non haemolysed serum is removed from the clot inactivated at 56 C for 30 minutes and two-fold dilutions made in physiological saline This represents the antigen to be tested against a constant dose of positive serum This test is of particular value in the rapidly fatal case in non vaccinated adults where death may occur before anything more than a scarlatiniform rash or perhaps before any rash at all has occurred

SMEARS OR SCRAPINGS OF MACULES OR PAPULES These are extracted with the minimal amount of physiological saline As the smears are frequently the only material available at this stage and cultures must also be done our practice is to place the slides in a petri dish add 0 6 ml saline to all the material available scrape the slides with needle or scalpel and leave at 4 C for a half to one hour remove 0 2 ml for three eggs and the remaining fluid is diluted with saline to 0 6 ml which is the minimal quantity on which to work for complement fixation test

VESICLE OR PUSTULE FLUID This is the ideal material for antigen The capillaries are placed in Ten Broeck grinders (closed tubes with glass rods with knobs at the bottom) and crushed Occasionally antigens made from such material particularly from pus are anticomplementary so that it is useful to treat first with a few cc of ether for 30 minutes The ether is then removed by pipette and evaporation and the required amount of saline is added and allowed to extract for 30 to 60 minutes If the same material is required also for egg culture 0 2 ml of suspension is removed and diluted with equal amounts of antibiotic

TOPS OF VESICLES AND PUSTULES AND SCABS These are the most time consuming to deal with but usually have a high concentration of antigen If one is presented with a rapidly fatal case with diffuse erythema and only a few doubtful early lesions all extraneous tissue must be removed from around the lesion and only the centre used for antigen As soon as the material is received in the laboratory it is placed in a dessicator and dried over calcium chloride for about one hour and then weighed The scabs are then ground in an enclosed tube such as the Ten Broeck grinder and several ml of ether added and left on for about 30 minutes The ether is then removed and the final grinding performed ten times the weight by the volume

Fig 2

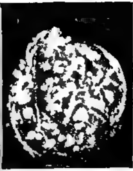

Fig 3

Fig 4

Fig 2 Variola virus Three day lesions on the chorioallantois
Courtesy A W Downie

Fig 3 Vaccinia virus Three day lesions on the chorioallantois
Courtesy A W Downie

Fig 4 Herpes simplex virus Three day lesions on the chorioallantois
Courtesy A W Downie

plasmic inclusion bodies in the mesodermal and ectodermal cells A herpetic lesion has a different macroscopic appearance (see Fig 4) and eosinophilic intra nuclear inclusions are present

The type of result one may expect to obtain in relation to the vaccination history of the patient is outlined in Tables 2 and 3 and may be correlated with the remarks in the paper by Downie on Pathogenesis The results which are of most significance in the early stages of the disease have been ringed It is obvious that specimens particularly of blood for laboratory tests

T ble 2

TESTS AND RFSULTS AT DIFFERENT STAGES IN PERSONS VACCINATED 3–40 YEARS PREVIOUSLY

	Pre erup		*M.P*		*I*		*P*		*Scab*		*Conv*	
Microscopy	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)				
Virus	0	0	0	+	0	+	0	—	0	—	0	0
Compl ment Fixat on												
Ant gen in Blood	∓	+	—	±	—	—	—	—	—	—	—	—
Antigen in Skin Lesions	0	0	0	±	0	+	0	+	0	+	0	0
Antibody in Serum	(+)	—	(+)	+	(+	+)	+	+	+	+	+	+
Vi us Cultu												
Blood	∓	+	—	∓	—	—	—	—	—	—	—	—
Skin Lesions	0	■	0	+	0	+	0	+	0	+	0	0

(a) = variola sine eruptione

(b) = modified to varying degree with rash

appearance of the pocks on the membrane of the fertile hen s egg A negative result based on insufficient material is of no significance but a positive result is definite A negative result with a known 1/100 dilution of vesicle fluid pus or scab is nearly 100% reliable A positive result in only the first one or two tubes is viewed with suspicion The usual result is a positive from ¼ to ⅓₂ (really ⅓ ₀₀)

When patients sera are being tested for antibody two-fold dilutions of serum are tested against an optimal dose of positive antigen A positive control serum will be included as well as a negative control antigen such as chicken pox

Egg culture The technique for the preparation of the fertile fowl s eggs is that used in most virus laboratories for the inoculation of the chorionic surface of the chorio-allantoic membrane Eggs which have been pre incubated at 37° C to 38 5° C from 10–14 days are commonly used The use of the egg for differential diagnosis of variola was suggested by Irons and his colleagues in 1941 - It is of particular value in patients who die before the appearance of a discrete rash and in the febrile pre-eruptive stage in contacts

Whatever the inoculum may be other than blood we always make it up to a minimum of 0 4 ml in broth containing 100 units penicillin and 50 units streptomycin per 0 1 ml and inoculate 0 1 ml onto each of three eggs *0 1 ml is also inoculated onto a blood agar plate and incubated* aerobically at 37 C The inoculated eggs are incubated at 37 C The three pox viruses variola (Fig 2) vaccinia (Fig 3) and cow pox produce lesions which can be distinguished macroscopically (The difference between vaccine and cow pox would only show up in a natural color picture) If the virus concerned is varicella there is no growth If the virus is vaccinia it is usually possible to make a diagnosis by 48 hours If the virus is variola a tentative diagnosis may be made if there is a heavy growth at 48 hours but the distinctive appearance of variola especially if there is only a small amount of virus possibly masked by inspissated serum is best seen after 72 hours incubation It will also be easier at 72 hours to differentiate between variola and herpes simplex Alastrim usually produces the same type of lesion as variola major although on two occasions once in Downie s laboratory and once at Colindale the lesions from an alastrim patient were too similar to herpes on primary isolation to give a definite answer A further passage produced lesions typical of variola Any membrane with doubtful lesions is examined histologically used as antigen in complement fixation test with antivaccinial serum and at the same time passed into more fertile eggs Histological preparations of variola and vaccinia reveal a concave lesion in the former and a convex lesion in the latter due to necrosis but the same type of microscopic intracellular reaction without a very definite inclusion body is seen in both By contrast a cow pox infected membrane shows a very haemorrhagic pock and highly acidophilic cyto-

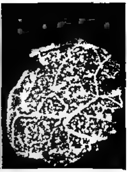

Fig 2 Fig 3 Fig 4

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The type of result one may expect to obtain in relation to the vaccination history of the patient is outlined in Tables 2 and 3 and may be correlated with the remarks in the paper by Downie on Pathogenesis The results which are of most significance in the early stages of the disease have been ringed It is obvious that specimens particularly of blood for laboratory tests

T ble 2

TESTS AND RESULTS AT DIFFERENT STAGES IN PERSONS VACCINATED 3–20 YEARS PREVIOUSLY

	Pre erup		*M.P*		*V*		*P*		*Scab*		*Conv*	
Microscopy	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)				
Virus	0	0	0	+	0	+	0	—	0	—	0	0
Compl ment F xat on												
Antigen in Blood	∓	+	—	±	—	—	—	—	—	—	—	—
Antigen in Skin Lesions	0	0	0	±	0	+	0	+	0	+	0	0
Antibody in Serum	(+)	—	(+)	+	(+	+)	+	+	+	+	+	+
V us Culture												
Blood	∓	+	—	∓	—	—	—	—	—	—	—	—
Skin Lesions	0	0	0	+	0	+	0	+	0	+	0	0

(a) = variola sine eruptione

(b) = modified to varying degree with rash

Table 3

TESTS AND RESULTS AT DIFFERENT STAGES IN PERSONS UNVACCINATED OR VACCINATED 20 OR MORE YEARS PREVIOUSLY

	Pre erup	*M P*	*V*	*P*	*Scab*	*Conv*
Microscopy	2–3 days	3–5 days	5–7 days	7–10 days		
Virus	0	+	+	0	0	0
Complement Fixation						
A (1) Antigen in Blood	(+)	(+)	(∓)	−	−	−
(2) Antigen in Skin Lesions	0	±	+	+	+	0
B Antibody in Serum	−	−	∓	+	+	+
Virus Culture						
Blood	(+)	(+)	∓	−	−	−
Skin Lesions	0	(+)	+	+	+	0

Pre erup = Pre-eruption V = Vesicular

M P = Maculo Papular P = Pustular

Conv = Convalescent

should whenever possible be obtained before suspected patients are vaccinated or re vaccinated Arbitrary periods of 3–20 and 20 or more years based on common experience were chosen for these two tables but of course variations in the size of the infected dose and the immune state of the individual may affect the response of the host at any time one year or more after a primary vaccination

In closing it might be advisable to remind pathologists who may be collecting specimens from patients with suspected smallpox that they should be vaccinated *before* going to see the patient The author also is of the opinion that virus pneumonia without a rash can occur after a 6–9 day incubation period following inhalation of virus by partially immune persons and this aetiology should be considered in exposed individuals with virus pneumonia while awaiting the results of tests for other possible virus agents such as influenza and psittacosis

Summary

A brief description is given of the type of tests available for the laboratory diagnosis of pox infections particularly in the early stages

The specimens required the manner of collection and a brief description of the techniques is outlined

These tests may be carried out by experienced virus workers and when the results are correlated with the clinical and epidemiological findings they may be of considerable value in controlling the spread of the disease

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25

The Diagnosis of Virus Infections Employing Tissue Culture Methods*

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Introduction

Only recently have the techniques of tissue culture been employed extensively as a method of diagnosis for certain viral diseases Many of the advantages and limitations of culture procedures as applied to this objective remain to be defined Yet already for example the application of *in vitro* methods in our laboratory has resulted in the development of simplified diagnostic procedures for the detection of infections with the poliomyelitis viruses and has permitted the serial propagation of agents previously not isolated such as those of varicella and of herpes zoster

In this paper developments in the field of the *in vitro* cultivation of the viruses pathogenic for man as related to possible diagnostic applications will be reviewed It is not our intent to emphasize details of methodology which at present are undergoing modification Consideration will first be given to applications of the culture procedure in the diagnosis of viral infections As different techniques are discussed examples indicating proven or potential utility of the method will be presented Lastly a few general considerations will be introduced regarding the tissue culture approach to the problem at hand namely the diagnosis and study of viral infections of man

Many investigators have utilized *in vitro* methods of cultivation for the study of mammalian viruses these contributions have been summarized by Robbins and Enders[1] and by Sanders Kiem and Lagunoff[2] Yet relatively few of these investigations until recently were directly referable to the

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problem of diagnosis Plotz and Reagan[3] in 1942 utilized cultures for the isolation of the street virus of rabies from the brain of both man and the dog The same year Sanders[4] employed cultures of mouse brain to stabilize the virulence of the virus of epidemic keratoconjunctivitis and later[5] reported isolation directly in tissue cultures However two considerations have delayed general utilization of the method First prior to the advent of the antibiotics long term maintenance of bacterial free cultures was difficult and the isolation of virus in tissue culture employing unfiltered inocula prepared from contaminated material such as human feces was not feasible The use of antibiotics to treat bacteria-containing inocula [6] or to eliminate bacteria when it is desired to employ contaminated tissues in the culture system[7 8] and finally the incorporation of antibiotics as routine[5] in the nutrient medium have permitted many of the recent applications of the culture method in the field of virology The second major consideration expediting utilization of the culture procedure has been the development of techniques for the identification of certain viruses under *in vitro* conditions In the early studies referred to identification of the agent propagated was usually dependent upon subinoculation into a susceptible host More recently methods which may obviate animal passage of agents isolated *in vitro* have been utilized These are based on elucidation of specific morphological or biochemical alterations produced by viruses in the tissue culture system—termed by us the cytopathic effect—or else on the utilization of culture virus as antigen in various serological tests Furthermore the specific inhibition by antibody *in vitro* of cytopathic activity or of the production of viral antigen permits in certain instances substitution of the culture for the embryonated hen s egg or animal in the performance of diagnostic neutralization tests It is now apparent that these methods developed in large part in connection with studies on the poliomyelitis viruses may be applied to the diagnosis of a variety of viral infections of man

Application of Tissue Culture Methods to the Problem of Diagnosis

In Vitro Isolation and Identification

Recognition of Virus by Cytopathic Activity The recognition of specific alterations induced by a virus in the morphology or metabolic activity of the tissue phase of a culture system is now an established technique for the detection of certain viruses *in vitro* In the evolution of procedures for detection of these specific alterations interest was first directed towards the demonstration of inclusion bodies Thus for example in the pioneering experiments of Steinhardt Israeli and Lambert[9] on vaccinia an unsuccessful attempt was made to demonstrate inclusions Later in the studies of Andrewes with virus III [10] of Rivers and coworkers with herpes simplex and vaccinia [11] as well as in those of Bedson and Bland[12] working with psittacosis the presence of cellular inclusions was utilized as evidence for

viral activity In general however the demonstration of inclusion bodies *in vitro* is applicable only to a restricted group of agents and is of limited value as a *diagnostic procedure* *Time-consuming technical manipulations* are involved Also it is appreciated that certain agents producing inclusion bodies *in vivo* do so with irregularity *in vitro* the observations of Bang Levy and Gey[13] on the growth of fowl pox are illustrative of this point Furthermore the interpretation on purely morphological grounds of intra cellular bodies particularly of the cytoplasmic variety is not without diffi culty For example we have observed in uninoculated tissue culture prep arations on occasion cytoplasmic bodies closely resembling those produced by certain viruses However we have not as yet observed non specific intranuclear inclusions and in the study of certain agents such as varicella the demonstration of this evidence of viral activity has provided valuable information

The recognition that *in vitro* certain viruses may give rise to gross altera tions in the cells in which they are multiplying has provided a technique of general diagnostic significance Plotz and Ephrussi[14] in 1933 noted in the case of fowl plague virus as did Ivanovics and Hyde[15] in 1936 work ing with virus III that infected cells failed to migrate or else degenerated Yet to Huang must go credit for observations of practical import on this phenomenon and its utilization for the titration and serological identification of a virus in the culture tube In 1942 Huang[16] observed that fragments of chick embryonic tissue infected with the western strain of equine enceph alomyelitis virus failed to grow applying this finding quantitative deter minations *in vitro* of infectivity and of neutralizing antibody were per formed In a second paper[17] the prediction was made that the phenomenon might be applied to other viruses particularly if tissues from man were employed The following year[18] Huang studied St Louis and Col SK viruses and noted that these agents while not cytopathic interfered with the destructive action of western equine encephalitis virus *in vitro* thus indi cating another method of titration in cultures Concurrently he also de scribed what we have since termed the pH differential test for the detection of virus *in vitro*[19]

The demonstration that the poliomyelitis viruses could be propagated *in vitro* in non nervous tissues[7,8] and likewise possessed cytopathic activ ity[7,20] has given impetus to the study of the cytopathogenic attributes of mammalian viruses Certain observations made in the course of studies on poliomyelitis will be summarized as illustrative of various techniques by which the cytopathogenicity of a virus may be established Our original observations were made with the suspended cell type of culture as recently reported in detail[1] Impaired metabolism of infected tissues as manifested by decreased acid production during propagation of the Lansing and Brun hilde strains in suspended cell cultures of human embryonic tissues was noted This difference between the pH of control and infected cultures

became apparent after relatively long periods of cultivation frequently between the 16th and 30th day It proved however to be a fairly reliable indicator of virus multiplication In practice we established arbitrary criteria for the pH differential test consisting of a mean difference of at least 0 2 pH units at the time of fluid change between the pH of the fluids in a minimum of three inoculated and in a minimum of three control cultures The pH differential test was applicable to the titration of viral infectivity Furthermore it was utilized as the indicator for the presence of virus in the first isolations *in vitro* of poliomyelitis virus from human fecal specimens Although the pH differential test has now been supplanted as a method for isolation of poliomyelitis virus by other tests for cytopathogenicity which are less time consuming this technique may be of value in studies on the diagnosis of other viral infections

Another indication of cytopathogenicity on the part of poliomyelitis virus was noted on histologic examination of tissue fragments removed from suspended cell cultures The degenerative changes observed in tissue fragments from cultures three weeks or more after inoculation were more extensive than those in comparable fragments from control cultures In the suspended cell system employed little cellular proliferation occurs and progressive degeneration of the tissue takes place Therefore it would appear that the histologic examination of tissues from suspended cell cultures may be of limited value as applied to problems of virus diagnosis Yet, for special situations the method may be useful Thus in attempts to isolate the etiological agent of varicella demonstration of intranuclear inclusion bodies in tissues from suspended cell cultures provided the initial evidence suggesting that a virus had been isolated [3] On the other hand it is probable that techniques based on the histologic examination of preparations obtained from cultures permitting of cellular multiplication such as the roller tube will be increasingly employed particularly in the detection of viral agents that do not possess a high degree of cytopathogenicity as expressed by rapid and more or less complete degeneration and death of the cell population

The third procedure utilized to reveal cytopathic activity of the poliomyelitis viruses consisted in the demonstration of inhibition of cell migration [9] from infected tissue fragments on transfer of such fragments from suspended cell to plasma hanging drop cultures Here again the method was time consuming for the inhibitory effect of virus on cell migration was not clearly demonstrable except in explants prepared from flask cultures maintained for at least 8 to 21 days At present this method is not invoked in work with the poliomyelitis group of agents However the procedure deserves further investigation for the study of agents that are not overtly cytopathogenic For example in experiments on the cultivation of mumps virus [4] we noted no cytopathic effect in cultures prepared with amniotic membrane from chick embryos Kilham and Murphy [5] employing cultures

of mouse embryonic tissue likewise observed no specific cellular changes Taylor [6] has now reported inhibition of cellular migration from fragments of chicken amniotic tissue following exposure to the virus of mumps although Watson[27] has not been able to confirm this finding

In an effort to demonstrate the cytopathogenic activity of the poliomyelitis viruses in a less tedious fashion attention was directed to a study of these viruses in roller tube tissue cultures [2] Employing techniques that have been described [8] it was ascertained that the introduction of virus into established cultures of a variety of human tissues was followed by gross destruction of the growing cells a phenomenon that could be observed readily on examination of the culture tube under low magnification The specificity of the degenerative process was made apparent by the fact that it was inhibited in the presence of homologous type specific antibody but not in the presence of heterologous antibody Thus specific identification of a poliomyelitis virus isolated in culture could be accomplished in a few days by transfer of the virus to subcultures in the presence of appropriate antisera Our findings were extended by other workers who demonstrated that monkey tissues likewise could be utilized for the cultivation of the poliomyelitis viruses [9 30] Subsequently modifications of technique directed towards simplification and standardization have been introduced In some laboratories slanted cultures[31 32 33] have been used Improved or simplified media such as those containing bovine amniotic fluid [34] hydrolysates of lactalbumin[32] or of bovine plasma [33] or else based on the synthetic medium of Morgan Morton and Parker[35 36] are now commonly employed A significant advance in the direction of providing a standardized source of human tissue for the propagation of viruses was made by Scherer Syverton and Gey[37] who showed that the HeLa strain of cells originally derived from an epidermoid carcinoma of the cervix would support the growth of poliomyelitis virus

It is in the study of those viruses that produce extensive cytopathic changes *in vitro* and in particular of the poliomyelitis group that the cultural method of diagnosis has most widely been applied In all probability few of the isolations of poliomyelitis virus accomplished in tissue culture have been described Yet indicative of work in progress are reports of the use of cultures of monkey testicular tissue by Riordan and coworkers[38] for the isolation of 24 strains and by Youngner and coworkers[39] for the isolation of 37 strains Scherer Syverton and Gey[37] have noted the isolation of 100 strains of poliomyelitis virus in cultures of HeLa cells In an extension of our original studies [40] 133 strains have been isolated in addition to the 13 previously reported [2] We have continued to employ various human tissues especially myometrium which is readily available and grows well in the bovine amniotic fluid medium The efficiency of the *in vitro* method of isolation from fecal specimens in this study has been of the order of 95% in patients with frank paralysis Attempts at isolation of

poliomyelitis virus from patients admitted with a clinical diagnosis of non paralytic poliomyelitis have been successful in about 65% of the patients studied It is apparent that certain of the failures in this group represent individuals that did not have poliomyelitis Indeed from some of these patients unidentified cytopathogenic agents have been isolated

It will next be pertinent to consider briefly certain other cytopathogenic viruses present on occasion in human feces Two such viruses were en countered in the course of our original study One agent which differed in cytopathic manifestations from the poliomyelitis viruses by an initial sparing of the peripheral outgrowth was shown to belong to the Coxsackie group This virus (Wieder) was classified as a new immunologic type (Boston) by Contreras Barnett and Melnick[41] and subsequently also has been isolated by Melnick and Agren[4] In our hands this type of C virus has exhibited cytopathogenicity for a variety of tissues[43] We and others have now isolated additional cytopathic Coxsackie viruses[44] Observations of this type suggest the desirability of a study of the behavior of the various Coxsackie viruses in cultures of human and of monkey tissues From present limited knowledge wide variations in cytopathic activity may be predicted Stulberg Schapira and Eidam[45] noted the marked cytopathogenicity of the Conn 5 strain for mouse fibroblasts With the antigenically related DeMole strain we also obtained growth in mouse tissue but failed with human skin and muscle tissue[43] although in the latter tissue the Boston type was highly cytopathic

The second agent that was not a typeable poliomyelitis virus is not overtly pathogenic for any of the common laboratory animals including the rhesus monkey[29] Other viruses lacking animal pathogenicity have since been isolated by the culture method both in Boston and by the Yale group[44] Steigman and coworkers[46] have likewise reported isolation of unidentifiable viruses from human feces and from the spinal cord of a patient with polio-myelitis[47] Thus a considerable number of cytopathic agents apparently not pathogenic for animals have been isolated in the course of studies on polio myelitis At present one may only speculate regarding the possibility that among these new viruses there is represented a fourth antigenic type of poliomyelitis virus that is pathogenic for man and not for the monkey

Note may here be made of yet other cytopathogenic agents encountered in human feces The virus of herpes simplex not uncommonly recoverable from stool material[43] has been observed to be highly cytopathic for human tissues *in vitro*[8] In our laboratory Neva[0] has recently obtained from the stools of 7 patients with an epidemic exanthem apparently new agents which produce morphologically similar cytopathic changes in cultures of certain human tissues

Our studies on the etiological agents of varicella and of herpes zoster[51] represent a different field of investigation wherein cytopathic changes have permitted recognition of the presence of an agent From the cutaneous

lesions of 7 cases of varicella and 3 of herpes zoster agents have now been isolated in cultures of human tissues that produce an unusual type of focal cytopathic change In affected areas the cells are swollen and almost uni formly contain intranuclear inclusions Serial transfer in culture has been successful only when tissue suspensions have been used as inocula but is then accomplished without difficulty This apparent failure of infectious material to appear in the fluid phase of the culture is a peculiarity of these agents which distinguishes them from those previously discussed A certain amount of confirmatory evidence relating to their etiological relationship has been educed from serological tests as will be noted shortly

Recognition of Virus by Detection of Antigen in Culture Material In con trast to the recent exploitation of the cytopathogenic phenomenon as an indicator of the presence of virus *in vitro* relatively few investigators have utilized tests based on the detection of viral antigen in cultures Yet several diagnostic applications are already apparent and may be adopted profitably in the study of agents that do not produce cytopathogenic changes of the poliomyelitis type

DETECTION OF COMPLEMENT FIXING ANTIGEN Complement fixing anti gen has been demonstrated by Maitland and Laing[3] in cultures of vaccinia virus and by Kunz [3] in cultures of mumps virus The demonstration by Svedmyr and coworkers[54 55] that the micro method of Fulton and Dum bell[56] was applicable to the detection of complement fixing antigens of poliomyelitis virus cultivated *in vitro* has provided a procedure of potential usefulness for the identification of unknown agents isolated in cultures Neva[60] thus has used the micro method to obtain evidence on the etiological significance of a virus isolated from a patient with an unidentified exanthem The same technique has been employed to investigate agents isolated from varicella vesicle fluid Convalescent phase sera from patients with chicken pox have been found to fix complement in the presence of culture antigen the specificity of this reaction is under investigation [57]

DETECTION OF HEMAGGLUTININS PRODUCED IN VITRO In 1948 we ob served that the viruses of mumps and influenza produced hemagglutinins on propagation in cultures of chick tissues [■] and the detection of hemag glutinins in cultures has now been employed by various workers to assay multiplication of these agents A method suited to large scale diagnostic studies appears to be that developed by Fulton and Armitage [58] Influenza virus was cultivated in fragments of chick chorioallantoic membrane em ploying a plastic tray with cups for 100 cultures Virus was demonstrated by the addition of red cells to the culture cups Other hemagglutinating agents such as the virus of fowl plague[59] likewise produce hemagglutinins in culture suggesting that this method for detection of antigen will have a place in the diagnostic armamentarium

THE DETECTION OF ANTIGEN BY APPLICATION OF FLUORESCENT ANTI BODY TECHNIQUES The introduction by Coons and coworkers[60 61] of spe

cific fluorescent immune serum as a histochemical indicator has opened up new approaches to the diagnosis of virus infections Employing fluorescein labeled specific antibody Watson[6] was able to demonstrate the localization of mumps virus in cultures of chick tissues Recently Coons and coworkers[61] have prepared a fluorescein anti human gamma globulin complex As applied to the detection of virus in tissue culture material infected cells are first layered with serum suspected of containing specific antibody after washing this is followed by exposure to the fluorescein antiglobulin complex and examination under the ultraviolet microscope Employing this technique we have noted in preliminary experiments brilliant fluorescence of focal lesions in tissues inoculated with varicella vesicle fluid material when the section has been treated with convalescent phase chickenpox serum and minimal or no fluorescence when treated with acute phase serum[63] from the same patient

Use of Tissue Culture in the Immunologic Diagnosis of Viral Infections

A major portion of this paper has been concerned with the consideration of phenomena applicable to the detection of virus in cultures Yet it is probable that the diagnostic laboratory will more often be concerned with the specific inhibition of certain of these phenomena than in their use as an indicator of the presence of virus on primary isolation

In vitro neutralization tests for the assay of specific antibody as measured by inhibition of cytopathogenicity of a virus have been most widely applied in the field of poliomyelitis research It is apparent that strains differ in cytopathic potentialities[64 65] and that selection of the test viruses is necessary Investigations on the variables involved in this *in vitro* neutralization test are in progress In one such study carried out by Ledinko and Melnick[66] the results of neutralization tests performed in cultures correlated closely with those obtained by the classical methods with the monkey and the mouse Already however application of this method has permitted accumulation of much valuable data Thus Salk and his coworkers[67] have assayed the antibody response in children following administration of inactivated poliomyelitis viruses Melnick and Ledinko[68] have determined the incidence of inapparent infection in a population group during an epidemic period

General Considerations on the Use of Tissue Culture Methods

In conclusion I wish to introduce a few general considerations pertaining to applications of the tissue culture method Already it is apparent that for the study of certain of the virus infections of man that manifest a high degree of host specificity the tissue culture tube may effectively for many purposes replace the human volunteer or the monkey The culture as noted almost 20 years ago by Cox[69] may be a more sensitive indicator of virus

than the experimental animal Illustrative of this point is the experience of Horstmann and McCollum[70] who obtained four isolations of poliomyelitis virus from blood in cultures and failed with the same material on inoculation into monkeys Even in those cases where the sensitivity of the two methods is essentially the same the culture technique may be manipulated to provide a more efficient indicator than the animal since it permits the testing of larger volumes of inocula than can be introduced into the living host by the appropriate route In contrast with the embryonated egg the prolonged periods of incubation that the culture affords may permit detection of agents that multiply slowly or become gradually adapted to growth in the new environment Considerations of this sort have justifiably stimulated *studies such as those of Henle and coworkers*[71] *on infectious hepatitis* and of Andrewes and coworkers[7] on the common cold

Yet it is well to stress the imperfect state of our knowledge regarding variables affecting multiplication of viruses *in vitro* In the past certain investigators have ignored the possible presence of specific antibody in serum employed as a constituent of the medium Little study has been made of the content of nonspecific viral inhibitor in culture components Andrewes [7] for example has noted an anti hemagglutinin for influenza virus elaborated in cultures of nasal tissue Our knowledge of cell types susceptible to the cytopathogenic effects of various viruses is fragmentary as is our knowledge of the optimum metabolic state of cells for the demonstration of this phenomenon In the case of the poliomyelitis viruses it is apparent that cytopathic manifestations appear when cells are nourished with a simple maintenance medium yet for the agents isolated from varicella vesicle fluid active cellular multiplication appears necessary for the complete development of specific morphological changes [7] Little attention has been paid to the possible concurrent propagation of non cytopathogenic viruses inadvertently derived from either man or constituents of the culture and to the possible interfering effect of such hidden viruses upon those under investigation It is also now apparent that one must take account of the possible presence of other types of pathogens in materials examined by the tissue culture method Randall and Hackney [3] have reported the development in tissue culture of *Histoplasma* from naturally infected human tissues We have recently observed that *Toxoplasma* will multiply freely in roller cultures of human tissues and in so doing produce a diffuse cytopathic effect [74]

In conclusion it should be emphasized that these precautionary remarks are made not in an attempt to depreciate the value of the tissue culture approach to the diagnosis of viral infections but rather to indicate the need for critical investigations in a rapidly developing field

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An Evaluation of Diagnostic Procedures for Virus and Rickettsial Diseases

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The remarkable advances in the field of viral and rickettsial diseases during the past few years have served to give the physician and the health officer an increasing awareness that potential assistance in the resolution of some of his problems is available In consequence there has been an ever increasing demand for such assistance and within recent years a small handful of laboratories whose primary mission is to provide diagnostic assistance has been established It is to be hoped that more laboratory facilities devoted primarily to diagnostic problems will become available In the case of serologic methods especially the techniques for the diagnosis of a number of diseases have been simplified to the extent that they can readily be undertaken by small public health and hospital laboratories and it would be desirable to have such laboratories incorporate at least some of the viral serologic tests into their sphere of activity The complexity of certain procedures however places them beyond the scope of the small laboratory and into that of a Central Reference Laboratory[1] whose functions require the trained personnel equipment and facilities that characterize a research laboratory

Because of various factors some of which I trust will become apparent as this discussion develops the philosophy of a diagnostic laboratory and its approach to a problem differ from that of a research laboratory It is not my purpose to discuss the problems of the research laboratory these are familiar to most of you It is my intention however to present for your consideration some of the problems of a diagnostic laboratory Although the tools are common to both their application and the methodology may be quite different Also a factor which may constitute no problem or a

minor one to a research laboratory may be a major one to a diagnostic laboratory

Microscopic Methods

Microscopic methods include the examination of fixed and stained tissue sections for the presence of pathologic change as well as the examination of imprint preparations and smears from tissues fluids exudates etc for the presence of inclusion bodies abnormal cells and even the agent

Histopathologic methods are little used since in general their value in establishing an etiologic diagnosis is quite limited Thus in the enceph alitides such methods are of no value in providing an etiologic diagnosis although they may serve a purpose in differentiating infectious processes from non infectious ones or necrotizing processes from the demyelinating ones seen in the post vaccinal type of encephalitis Of the few instances in which these methods serve to reveal etiology there comes to mind almost immediately the examination of brain tissue for rabies virus infection and the examination of liver tissue obtained at post mortem or by viscerotomy for yellow fever Histologic methods are also useful in the examination of tissues from animals used in virus isolation work e g the Coxsackie group of viruses In the latter instance examination of the brain muscle and pancreas serves to give a rough classification of the agent as to group if more detailed or precise information is required the agent must be typed by serologic means

As to microscopic examination of imprint preparations or smears this finds a somewhat wider application but the conclusions reached through examinations should be supported or confirmed by another method usually isolation of the virus This is in addition to the usual admonition that the interpretation should be based on a consideration of the epidemiologic and clinical data information which in my experience at least a virus reference laboratory only seldom receives Perhaps the widest use of the microscopic technique is in its application to rabies a condition which existent or suspected provides the laboratory with a large amount of material for direct examination However because errors in interpretation can occur even in experienced hands and because occasional preparations apparently contain no Negri bodies although the virus is present in the tissue confirmation of all interpretations is sought through routine inoculation of the specimens for virus isolation

The presence of herpes simplex virus in lesions of the oral cavity pre sumably may be detected through the presence of intranuclear inclusions in cells obtained through scrapings from lesions of the gingival or buccal mu cosa Blank and his associates[1] have confirmed Tzanck s observations[2] that a virus type" multinucleate giant epithelial cell is present in scrapings taken from the peripheral portion of vesicles produced by the viruses of herpes simplex herpes zoster and varicella These pathognomonic cells apparently

are not found in vesicular lesions of the skin due to any other cause, and their presence rules out variola and vaccinia Additional laboratory procedures are required to differentiate between herpes simplex and herpes zoster infections It has also been reported that differentiation between the elementary bodies of herpes simplex herpes zoster and varicella is possible by means of the electron microscope I doubt however that many diagnostic laboratories will be able to justify acquisition of an electron microscope for such a purpose

Differentiation of variola from varicella or its recognition when it occurs in the vaccinated person is not always simple on clinical grounds alone and the laboratory is frequently called on for assistance The value of stained smears from skin lesions has been pointed out by van Rooyen and Illingworth [3] The method is applicable primarily to smears prepared from papules and from vesicle fluid the results with pustular material are less reliable because of the obscuring debris The usefulness of the method depends upon the careful preparation of the smears a factor of which few physicians are aware The correct interpretation of the findings requires some experience Even so the interpretations must be evaluated on the basis of the patient s clinical data and his epidemiologic background Moreover as Downie emphasizes confirmatory methods should be employed [4]

In addition to the agents of variola and vaccinia the other larger viruses and rickettsiae may often be detected by direct microscopy Thus in the case of birds suspected of ornithosis smears prepared from the enlarged spleen or the air sac may reveal the presence of the virus Similarly examination of pus from the buboes of lymphogranuloma venereum may be helpful Here too direct microscopy should be regarded as an ancillary method and should not replace isolation of the virus

Isolation Methods

The isolation and identification of an agent is a rather protracted procedure Because of this and because of the heavy demand it places upon the time of technical personnel it is avoided insofar as is possible in routine diagnostic work A further objection is that from the diagnostic standpoint it seldom gives any more information than can be obtained by serologic methods which are simpler and much less costly

There are of course some conditions under which isolation of the agent is desirable One such condition has already been mentioned viz the need for confirmation of interpretations based on microscopic methods Also attempts at recovery of an agent are almost mandatory in trying to reach a diagnosis in obscure or vague conditions of presumed viral etiology Finally virus isolation attempts are frequently desirable in attempting to resolve the problems confronting a health officer in the presence of an outbreak of unknown etiology The latter situations represent in essence research problems but they must be undertaken if we are to learn anything

of the etiology of diseases of uncertain causation and if we are to discover new agents which can eventually be employed for diagnostic purposes

It has been stated that in a few diseases the isolation and identification procedures are so simple that diagnosis of the infection can be made even during the patient's illness i e within a matter of a few days to a week This leads the clinician and the health officer to assume that such a procedure is feasible under all conditions and circumstances and it is difficult for him to comprehend the objections raised by the laboratory when it is suddenly confronted with the examination of hundreds of specimens Undertaking isolation of the causal agent in a suspected disease such as influenza poses a number of practical problems First of all even though the individual may have influenza as subsequently proved by serologic tests the virus may not be present for one reason or another in the throat garglings as received in the laboratory before this is ascertained however at least two and perhaps three passages will have been made in embryonated eggs In accordance with the dictum that negative findings have far less significance than positive findings (although the significance of the latter must now be qualified in the light of recent observations) failure to recover the agent does not rule out the disease Secondly since identification of influenza virus usually is based on the appearance of hemagglutinins not infrequently several passages are required before the hemagglutinin titer attains a sufficiently high level to permit identifying inhibition tests to be conducted Even when the complement fixation method is used several passages may be required before the virus produces a sufficiently potent antigen to permit its identification Finally material submitted to a diagnostic laboratory cannot be stored for examination at leisure or when opportunity affords but must be examined on a current basis Consequently when many hundreds of specimens are to be examined over a matter of a few weeks or a few months the task confronting the laboratory in the light of the above considerations is one of no mean magnitude

If virus isolation is undertaken certain requirements must be met to weigh the balance in favor of success These requirements usually are stated in some general manner such as (a) the proper material (i e material in which the virus is generally found) must be collected at the proper stage of the illness (b) the viability of any agent which may be present in the material should be protected from the time of collection to the time of inoculation and (c) the material must be inoculated into a suitable laboratory host Although the meaning of these generalities apparently is clear when they are read casually questions arise when one attempts to put them into practice

A general indication as to when materials should be collected is given in Table 1 which is adapted from Horsfall and lists those periods in relation to the evolution of the illness during which the virus might be expected to be present or to be absent From the standpoint of clinical medicine the

Table 1

RELATION OF STAGE OF ILLNESS TO PRESENCE OF VIRUS IN TEST MATERIALS AND TO APPEARANCE OF SPECIFIC ANTIBODY

Period of illness	*Virus detectable in test materials*	*Specific antibody demonstrable*
Incubation	Rarely	
Prodromal	Rarely	
Onset	Frequently	
Acute phase	Frequently	Frequently
Recovery phase	Rarely	Generally
Convalescent stage	Very rarely	Usually

Adapted from Horsfall

period of incubation can be omitted from consideration since it is only under very unusual circumstances that the patient is seen at this stage of the infection The prodromal period too can be dismissed for similar reasons Usually the patient is first seen by a physician at the time of onset or shortly thereafter and it is at this time that material is collected for examination if a viral infection is suspected The usual experience has been that the agent is detectable during the early stages of the illness but not when recovery is under way or essentially complete The division of an illness into an acute and recovery phase is not susceptible of rigid separation on a temporal basis and the practicing physician cannot determine in any given illness when the most propitious time for the collection of material has passed More precise information as to the number of days post onset it is possible to detect virus in a given material for a particular disease would be helpful This would not only increase the chances for successful recovery of the agent but would help eliminate much of the useless testing to which a laboratory is subjected We might also point out that an analogous situation exists with respect to blood specimens for serologic studies Most patients are not seen or clinical suspicion is not aroused for a variable number of days after the onset of the illness It is difficult in such instances to decide whether the first blood specimen obtained represents an acute phase or a recovery phase specimen and to decide when a second specimen should be taken or even whether it can be expected to show anything if it is taken Here too more precise information is needed as to how soon antibody appears how rapidly it rises the maximal levels attained and the period of persistence It is true that a certain amount of information on these two aspects is available in the literature but the observations are too few so far as I am aware to permit a statistical evaluation

Problems are associated too with respect to the type of material to collect for examination In the case of the patient whose illness terminates fatally this is relatively easily answered since the tissues which form the

site of predilection for the virus represent the material of choice Furthermore additional tissues may be taken for extra good measure As for the living patient the presence of virus has been demonstrated in the throat washings of patients with such diseases as poliomyelitis influenza mumps and herpangina in sputum in individuals with psittacosis or the so-called viral pneumonia in the saliva in cases of rabies and in the stool of individuals with poliomyelitis In other diseases the virus has been recovered from the blood as for example yellow fever dengue Colorado tick fever Venezuelan equine encephalomyelitis and the rickettsioses In the central nervous system infections submission of cerebrospinal fluid is almost invariably recommended It is true that a number of agents have been isolated from cerebrospinal fluid but the number of such positives reported is hardly impressive and it would be interesting to know what the proportion of positives would be if the total experience were recorded In the light of our experience I assume that the successful recoveries reported in the literature were the result of work done under ideal conditions Such conditions obtain only when the diagnostic laboratory is an intimate part of the hospital or is located nearby Our laboratory receives material from the entire state and we have yet to isolate a virus although over the past few years we have tested many hundreds of specimens of spinal fluid We have therefore come to the conclusion that insofar as a Central Virus Reference Laboratory is concerned spinal fluid is a poor source material for virus isolation attempts Finally there remains that comprehensive category of diseases whose nature is so vague or obscure that diagnostic assistance is urgently sought It is in this group especially that one encounters difficulties as to the type of material which should be examined If in order to be sure that all possibilities are covered a variety of materials is collected the laboratory soon finds itself conducting essentially a research problem on each such patient

It appears to me that it would be worth while to re-examine the pathogenesis of many virus diseases with the view of determining more closely the routes of excretion of the virus and of applying this knowledge to diagnosis Let us consider for a moment infections of the central nervous system It is stated that in lymphocytic choriomeningitis and in St. Louis encephalitis the virus is present in the cerebrospinal fluid Yet as I have pointed out above we have never been able to isolate any viral agent from this source Similarly it is stated that viremia is commonly present in lymphocytic choriomeningitis and in Venezuelan equine encephalomyelitis but not in Eastern or Western equine encephalomyelitis St Louis encephalitis or Japanese B encephalitis On the basis of such information virus isolation attempts in the case of lymphocytic choriomeningitis and Venezuelan equine encephalomyelitis should be directed at the blood stream in the case of the other diseases this would apparently be pointless Consequently the only recourse left in these latter diseases at least under the

operating conditions of a reference laboratory such as ours is to conduct serologic tests It occurs to me that it might be of value to study more intensively the possibility that viremia exists in some of these diseases Similarly since Venezuelan equine encephalomyelitis virus has been recovered from the throat washings of patients[6 7] the possibility that the causal agents of such diseases as Western and Eastern equine encephalomyelitis and St Louis and Japanese B encephalitis might also be detectable in throat washings deserves investigation

Assuming that appropriate material for study has been taken and that it has been taken at the appropriate stage of the illness due care must be taken to insure the viability of any agent which might be present Under those conditions in which the material can be subinoculated rather promptly into suitable laboratory hosts this requirement presents no problem When material is sent to a laboratory at some distance however success is often jeopardized by failure to observe this point It is generally accepted that the specimen should be placed under refrigeration as soon as it is taken and that if it is to be sent any distance it should be shipped under refrigeration Dry ice is always recommended because it produces a very low temperature and is used for the storage and preservation of viruses in the laboratory In many areas dry ice is not obtainable and in such cases we recommend the use of wet ice although this apparently is unorthodox Shipping instructions sometimes direct the use of a thermos jug which is not very practical for a number of reasons—few physicians have these available for one thing but the paramount objection is the high loss from breakage We have found that one of the most practical methods of shipment is to place the well sealed specimen container into a small carton containing cut or crumpled paper and to place this carton within a larger cardboard box containing sufficient dry ice to keep the specimen frozen during the journey and plenty of cut or crumpled paper (or wood shavings) to act as a shock absorber as the dry ice evaporates * I should like to emphasize that while shipment under refrigeration may represent the ideal step it is not a very practical procedure in applied diagnostic virology since few physicians have the time or the facilities to ship specimens under either dry or wet ice refrigeration Years ago a solution of 50% glycerin in buffered saline was used for the preservation and storage of viruses and it would be desirable to investigate the use of this or a similar menstruum for the shipment of viral agents without refrigeration

This brings us to the third requirement viz subinoculation of the collected material into suitable laboratory hosts The variety of laboratory hosts available is not large and their use is governed by their availability and cost I do not propose to discuss the virtues and the faults of each

* An excellent mailing container is the type used by the Army Medical Service Graduate School, and manufactured by the Hollinger Corporation Arlington Virginia It is made of fibreboard is well insulated and is surprisingly light

laboratory host but rather have selected several which will illustrate the differences in approach to a problem by a diagnostic laboratory and by a research laboratory and why certain factors which are of no great moment to the latter are highly important considerations to the former Thus no diagnostic laboratory with its limited budget and personnel could operate very long if it attempted the use of monkeys as laboratory hosts for routine examination Nevertheless numerous stool specimens are submitted from cases of suspected poliomyelitis especially during the period of highest prevalence of this disease The physician is aware through the literature that isolation requires the use of monkeys but is unable to see that a line must be drawn between diagnosis made on a single individual for clinical purposes and diagnosis made on groups of individuals for the purposes of epidemiologic studies

The mouse is a basic laboratory animal and in addition to susceptibility the factor of age is of importance It is now well known that suckling mice are highly susceptible to a number of agents to which adult mice are completely or almost completely resistant Thus the use of new born mice has served to uncover a whole new group of agents pathogenic for these very immature animals On the other hand it is possible that an agent may grow better in the mature than in the young animal as for example is true of the Lansing strain of poliomyelitis virus although the latter admittedly has had a long period of adaptation to the adult mouse Because of such considerations we use both new born and adult mice for virus isolation purposes This takes into account the fact that newly isolated viruses do not always behave according to the description of pathogenic properties given in text books descriptions which are based on experimental work employing highly adapted laboratory strains Our experience with such a situation concerning the St Louis encephalitis virus has already been recorded [8] By way of further illustration we might use the herpes simplex virus Descriptions of this agent characterize it as producing plaques on the chorio-allantoic membrane of the embryonated hen s egg and keratitis on the rabbit cornea In our experience newly isolated strains may or may not produce plaques and sometimes adaptation through prolonged passage is required for plaque production Similarly inoculation of the cornea does not invariably produce a keratitis and we do not rule out herpes simplex virus because a keratitis does not develop instead the inoculated rabbits are bled after ten to fourteen days to determine if herpetic antibodies have appeared

The use of the developing chick embryo[9] represents a basic tool in virology and its value and versatility have recently been reviewed in an excellent article by Cox [1] Various routes of inoculation can be used for special purposes but the amniotic and the yolk sac routes probably are those most commonly employed for isolation work There is a variety of techniques for inoculation and for passaging but I should like to point out that only the simplest are the most useful in large scale work The window tech-

operating conditions of a reference laboratory such as ours is to conduct serologic tests It occurs to me that it might be of value to study more intensively the possibility that viremia exists in some of these diseases Similarly since Venezuelan equine encephalomyelitis virus has been recovered from the throat washings of patients[6,7] the possibility that the causal agents of such diseases as Western and Eastern equine encephalomyelitis and St Louis and Japanese B encephalitis might also be detectable in throat washings deserves investigation

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The use of *in ovo* neutralization tests has been associated almost entirely with special studies The method has found little acceptance in diagnostic laboratories

In ovo neutralization tests have employed three types of endpoint for the determination of the neutralizing activity of sera The first of these end points is based upon the ability of the virus to produce plaques or pock like lesions on the chorio allantoic membrane The degree of neutralization which occurs is based on the quantitative reduction in the number of virus particles capable of producing lesions which is indicated by the number of plaques produced The technique is time-consuming requires considerable experience and is open to a number of errors which have been discussed by Burnet and Faris [15] Estimation of the infective titer alone is apt to yield somewhat variable results and in the presence of serum an additional error is introduced into the method by the lack of proportionality between virus dilutions and plaque counts [16]

The chorio-allantoic membrane technique does not appear to be as well suited for the quantitative measurement of antibody as does a simpler technique based on a lethal endpoint Inasmuch as determination of the neutralizing capacity is based on the death or survival of the embryo some what larger numbers of eggs must be used than are required by the pock counting technique However infections with virtually all of the viruses which produce lethal effect in the chick embryo are much more readily diagnosed by *in vitro* serologic methods

The third test which has been widely used in work with those viruses which produce chick cell hemagglutinins has an endpoint based on the appearance or non appearance of hemagglutinins in individual eggs inoculated with the serum virus mixture In this test a second set of variables i e the presence or absence of hemagglutinins is introduced and adds to the inherent variation already present in the method Examination of each egg or group of eggs for the presence of hemagglutinins represents an undesirable addition to the work load and the information derived from this test can be obtained more readily by *in vitro* tests

As concerns animals a number of species have been and are used for neutralization tests but the albino Swiss mouse is almost the *sine qua non* for neutralization tests in the diagnostic laboratory Selectively inbred strains of a high and uniform susceptibility should be used In addition the age of the test animal is of importance under some conditions With neurotropic agents both suckling and adult mice are about equally susceptible to cerebral infection the suckling mice being on the whole perhaps slightly more susceptible Some of these agents however will infect only suckling animals when a peripheral route of inoculation is used This property has been utilized in the extraneural neutralization test which has been shown to be far more sensitive than the usual intracerebral method [17,18] On the other hand there are agents such as the dengue virus and the Coxsackie

nique for exposure of the chorio allantoic membrane or similar exposure of the allantoic or amniotic cavities requires time Consequently, our standard operating procedure consists of direct inoculation into the chosen site without any preliminary exposure Direct exposure is done only occasionally and then for special purposes such as plaque counts Similarly time is not taken to harvest and process the whole embryo or a part of an embryo when the fluids alone will suffice

The recent development by Enders and his associates[11] of a tissue culture technique of the fixed cell type for the propagation of poliomyelitis virus represents an important and an outstanding contribution Since its introduction the method has been repeatedly modified and simplified by many workers and the development in this field is well presented in a recent article by Weller [12] Despite the simplification already achieved the method is still too unwieldy for application to routine diagnosis on a large scale The present methods place a disproportionately heavy burden on the time of available personnel i e the number of examinations which can be made are too few in relation to the heavy expenditure of time involved and the method must be made considerably less cumbersome before it can be adopted as a routine diagnostic tool The same is true of the recent technique described by Scherer and his colleagues[13] in which the tissue element consists of human malignant epithelial cells This technique however has the merit of utilizing cells which can be propagated indefinitely *in vitro* and thus removes dependence upon human embryonic or surgical tissue with the attendant difficulties of maintaining a continuous supply and on monkey tissues which are relatively costly

Serologic Methods

By far the greatest proportion of examinations conducted in a diagnostic laboratory consist of serologic tests I would without hesitation place the complement fixation test first as the most useful of the several methods and the agglutination test with specific antigens as the least useful The neutralization and hemagglutination inhibition tests come in between but a decision as to which is the more useful is difficult to make the neutralization test is applicable to more diseases than is the hemagglutination inhibition test although the latter possesses the virtue of being in many instances simpler and less expensive than the former From the diagnostic standpoint the hemagglutination inhibition test is applicable to few diseases for which there is not also a complement fixation method available

Neutralization Test This test might be considered first since it dates from the time of Sternberg s work with vaccinia virus in 1892 [14] was used for many years before adequate *in vitro* methods were developed and represents the criterion by which the specificity and adequacy of other methods has been assessed The test can be conducted in embryonated eggs in laboratory animals or in tissue cultures

While the age of the embryo may as in the case of mice be an important factor in the sensitivity of the test system this is not necessarily true under all conditions It has been found [21] for example that the greatest degree of neutralization of Eastern equine encephalomyelitis virus in 15 day-old embryonated eggs occurs when these are inoculated by the yolk sac route and the least amount occurs when inoculation is performed by the chorio allantoic route difference in the sensitivity of the two routes is the responsible factor here and not the age of the test animals The factor of age however is indicated in the fact that 11 day old eggs for example show intermediate degrees of neutralization when inoculated by either route [1]

The route of inoculation also affects the end results There is the well known observation that mixtures of vaccinia virus and immune serum may be non infective in the rabbit on intracutaneous inoculation but are fully infective on cerebral inoculation or on inoculation onto the chorio-allantoic membrane of the embryonated egg Similarly quantitative neutralization tests with influenza virus show that many mixtures of virus and immune serum which are non infective in the mouse are infective in the chick embryo [23] Likewise a greater apparent degree of neutralization of influenza virus is obtained on the chorio allantoic membrane than is obtained when the allantoic cavity is used [24] Apparently such results are a reflection of the differences in the amount of unneutralized virus required to infect one host as compared with another and of the susceptibility of the host to infection by different routes

Whether incubation of serum virus mixtures is necessary is still a point of some debate Some workers claim that incubation produces a firmer virus antibody union and thus enhances the neutralizing effect Others however claim that maximum neutralization of virus by antiserum is achieved within a matter of moments and that incubation produces only varying degrees of secondary inactivation of the virus This difference of opinion is not due entirely to the use of different methods and techniques but has arisen even in instances where essentially similar procedures have been used

It is thus obvious that many factors underlying the neutralization test have yet to be clarified Some contributions to this subject have appeared during the past few years and show that the quantitative relationship between virus and immune serum is markedly influenced by a number of variables The most important variables appear to be the host-cell system employed and the route of inoculation used [3] The findings of Horsfall and his co-workers indicate that if quantitation involves the use of materials containing large amounts of antibody a host-cell system highly susceptible to infection should be employed [3] If as is true in diagnostic work quantitation is directed at the detection of small amounts of antibody or of small increases in the amount of antibody only comparatively small virus doses should be used and in addition the host cell system should be one which is relatively insusceptible and thus permit infection to supervene only when

viruses which are pathogenic by the cerebral route only for the very immature mouse and not for the mature animal and consequently neutralization tests are feasible only in the former

The usefulness of the baby mouse has not been fully explored although it is recognized Objections to the use of the baby mouse for neutralization tests (and for isolation procedures too) have been expressed to the effect that resistance to extraneural injection may develop irregularly and hence a larger number of mice must be used also to obtain mice of a known age large expensive breeding colonies are required The objection to the maintenance of breeding colonies to provide infant mice has been overcome in large part by the value of these animals in virus isolation work e g the Coxsackie group of viruses and in the production of superior antigens not otherwise obtainable e g complement fixing antigens for dengue and for poliomyelitis (MEF–1 strain) As to the use of larger numbers of mice I think a more serious objection than that of irregularly developing barriers is to the cannibalism that frequently disrupts tests and requires a repetition if as is suspected by some this is due to dietary factors perhaps a remedy can be easily evolved

The variety of methods by which the neutralization test is performed is surprising Part of the variation might be expected e g differences in hosts because of their differences in susceptibility and differences in the route of inoculation because of differences in pathogenetic properties between agents Some techniques have been devised for special purposes such as providing higher accuracy in a special study or to permit large scale examination of materials in special surveys or epidemiologic studies Consequently the tests differ as to the ratio of virus serum volumes employed whether or not incubation is used and if it is there are differences in the time and the temperature employed Last but not least is the matter of using varying virus-constant serum versus constant virus varying serum dilutions The net result is the existence of a welter of methodology out of which we should make some serious attempts to bring order

A comparative evaluation of the various methods is difficult because of the diversity of techniques employed not only between viruses but even with the same virus Differences may be encountered in the same host with the same virus if different methods are used Thus using the chorio allantoic membrane route with herpes virus the results of one group of workers indicated that human sera contain either no demonstrable herpetic antibody or else a very high level [8] no intermediate levels were encountered This all or none character of neutralization was not encountered by other workers [9] who used the yolk sac route of inoculation and tested varying dilutions of serum against a constant amount of virus The differences in the two procedures lay in the age of the embryo the route of inoculation and the use of constant virus dilutions in one case and of constant serum dilutions in the other

unheated normal serum to inactivated test sera to enhance neutralizing effect may give rise to peculiar results which are difficult to interpret

The use of very low storage temperatures for the preservation of *specific* antibody however may represent a different situation but there is very little precise information as to the exact value of such a procedure There have been only a few studies on the comparative stability of antibodies at different storage temperatures and these moreover have been concerned with relatively few specimens stored for relatively brief periods of time Additional studies employing sufficient numbers of specimens to give results whose significance can be statistically evaluated are needed

There is also the matter of the appropriate virus strains to be used The tendency is to employ well known well adapted strains which usually have a high passage level and quite conceivably are antigenically different from strains which have been freshly isolated and have few animal passages For example we have observed that the Winkler strain of St Louis encephalitis virus gives more clear cut results in neutralization tests than does the Hubbard strain Others[29] also have observed differences between strains of the St Louis encephalitis virus There is also evidence that antigenic differences exist between strains of herpes simplex virus In neutralization tests it would seem as desirable to use strains with a broad antigenic constitution as it is in the influenza hemagglutination inhibition test

Tissue culture methods have served for the assay of poliomyelitis antibodies but these are still essentially research methods and are confined to epidemiologic studies Further simplification of the method is necessary as is information on its scope and limitations The current methods are too costly for routine application not only in terms of the ingredients but also in terms of the personnel time involved The great simplification which has already been achieved indicates that techniques applicable to large scale use should soon be available However intensive work on the development of a complement fixation method should continue as such a method would have numerous obvious advantages over a cultural one

Complement Fixation For large scale work the complement fixation test represents the simplest and the most satisfactory method There is a variety of techniques the differences lying in the concentrations of reagents used the volumes of each employed the time and temperature of the primary incubation period and the length of the secondary incubation period before readings are made The variations which have arisen over past years in part are derived from efforts to avoid difficulties engendered by impure and insensitive antigens and in part express personal preferences in methodology There is no real need for many of these differences today since fairly good or excellent antigens are available for many of the diseases for which tests are conducted The diversity of methods is apparent in reading the literature or compends on diagnostic procedures covering the commoner viral and rickettsial diseases A diagnostic laboratory cannot undertake the use

comparatively large amounts of unneutralized virus are present in the test mixture

Important information has also been added to the problem of the use of constant virus varying serum versus the constant serum varying virus techniques time does not permit of full development and exposition of this subject and the interested reader is referred to a recent paper by Tyrrell and Horsfall [3] In essence it was observed that if the slope of the neutralization line which represents the relationship between the logarithm of the amount of virus neutralized and the logarithm of the serum dilution end point is greater than 1 0 a large change in virus titer is required to reveal small differences in antibody concentration if the constant serum varying virus method is used For example in a virus antibody system with a slope of 3 0 a 1 000 fold change in virus titer reflects a ten fold increase in antibody concentration In contrast the constant virus varying serum method is superior in several respects Measurement of antibody levels is more accurate because even large variations in the amount of virus used produce only small changes in the titer of the serum Also a wide range of serum dilutions can be examined and the method permits the use of viruses with low titers We hope that the application of these principles will result in the development of tests that are far more satisfactory for diagnostic purposes than are the current procedures

I should like to take a few moments for other items that are associated with neutralization tests It is widely accepted that sera for neutralization tests (and for other serologic tests also) should be removed from the clot promptly and stored in the frozen state This is on the basis that the neutralizing capacity falls if the serum is stored at the usual icebox temperature of 4° to 6° C the end result being that the neutralizing capacity of a stored acute phase blood may be lower than that of a relatively fresh convalescent phase blood and thus indicate that a rise in antibody titer apparently occurred in the interval between the two bleedings Morgan and Whitman [8] using Western equine encephalomyelitis antisera showed that sera stored in the frozen state had a higher neutralizing capacity than sera which had been stored at 4 C or had been heated By adding fresh guinea pig serum Morgan could restore the activity of sera that had been stored at 4 but not of sera which had been heated Whitman could restore the activity of both old and of heated sera by means of fresh guinea pig monkey or human serum (A literature summary is given by McCarthy and Germer) [7]

Actually this heat labile factor is a non specific virus inhibitor present in normal sera and active against a number of agents [7 25] One has his choice of preserving this non specific neutralizing factor by storage of the serum in the frozen state or of destroying it by heating at 56° C for 30 minutes We are inclined to agree with Ginsberg and Horsfall [8] that it is hazardous to employ unheated sera in neutralization tests (and in other serologic tests too for that matter) these authors also point out[25] that the addition of

produce the desired concentration is computed Suspensions prepared by this method are said to be reproducible both as to accuracy of concentration as well as susceptibility to lysis

The sensitivity and specificity are also affected by concentration of the reagents used and by the physical conditions of the test An example of this has been provided by Schubert et al[11] who compared five complement fixation techniques for the diagnosis of the rickettsioses Their findings bore out the well known fact that sensitivity and specificity are to some degree inversely related The most sensitive method of the five was found to be the least specific and the least sensitive method was found to be the most specific The other three methods fell in between Preference was given to one method (the modified Kolmer) because of the balance between sensitivity and specificity and also because the procedure is widely known The highest degree of sensitivity compatible with a high specificity is desirable since one is dealing with individuals as patients and not with individuals as an epidemiologic group Overnight incubation in the cold increases sensitivity as compared with brief incubation at 37 C and hence is employed for this reason as well as for convenience Tests with a primary incubation period of one hour have been advocated for rapid diagnosis or emergencies This only serves to perpetuate the widespread but erroneous belief that an unequivocal diagnosis can be made on the basis of a diagnostic titer obtained through examination of a single specimen of serum

A few words on the subject of non specific and anticomplementary reactions may be pertinent Non specific reactions are encountered in a variable proportion of sera In some cases these reactions are due to antigenic relationships between the test antigen and the control antigen In such instances these reactions can be avoided by awareness of the existence of antigenic relationships between certain viruses or groups of viruses Non specific reactions may also be due to the presence of unknown tissue components in the antigen and the use of acetone or benzene-extracted antigens serves to remove much of this difficulty In some instances the reactions appear due to a heat stable non specific substance in the serum[3] [33]

As to anticomplementary reactions these can be divided into two groups viz intrinsic and extrinsic The intrinsic group comprises those sera in which the anticomplementary activity is an inherent property of the serum of the individual and is not due to faulty technique In some individuals repeated bleedings will give sera which are anticomplementary on every occasion whereas in others the serum may be anticomplementary on some occasions but not all Lighter[34] found that 88% of 144 patients who gave repeated anticomplementary reactions had syphilis and suggests that when ever such reactions are encountered the possibility of syphilis should be inquired into The extrinsic group comprises those sera in which the anticomplementary action is caused by extraneous factors viz bacterial or

of a variety of methods for at least two reasons viz the volume of material to be handled requires a uniform method for all the diseases and in addition the possibility of errors is greatly increased when a number of variations of the same basic method is used The use of a standard procedure acceptable to all laboratories is highly desirable but until workers are ready to sacrifice their personal preferences I doubt that standardization will soon be achieved

The sensitivity and the specificity of the complement fixation test are affected by the accuracy with which reagents are standardized in relation to each other In essence the test consists of five variables three of which—antigen complement and hemolysin—are assayed for use in the test on the basis of a fourth variable sheep erythrocytes which are used directly in the test The final procedure is titration of the fifth variable the serum under examination Consequently the complement fixation test is open as is any system of variables to an over all error greater than the expected error due to each of the variables [30]

Titration of the antigen represents one of the variables Two procedures are in common use viz the so-called straight line and the checkerboard or box titration In the straight line procedure falling dilutions of antigen are tested against a single dilution of immune serum the box titration employs falling dilutions of antigen against falling dilutions of the immune serum The former method gives a maximum dilution of antigen that will fix with an arbitrary dilution of serum whereas the latter gives the highest dilution of antigen that will fix with the highest dilution of serum and thus establishes what is essentially the optimal proportion between antigen and antibody The unit as determined by the box titration therefore is one that can be expected to give an amount of antigen that will react with very low concentrations of antibody whereas the straight line method gives a unitage which may be inadequate to do so

The sheep erythrocyte suspension comprises one variable to which less attention has been paid as compared to the others although as Collier et al[30] have pointed out it is an important one Preparation of the suspension requires some care to prevent alterations in the cells which may affect the test Suspended cells can be made accurately to the desired concentration by packing to a minimum volume This procedure may produce obvious destruction as is indicated by visible hemolysis More important however is the change in physico-chemical properties which makes the cells more susceptible to hemolysin This latter alteration can be demonstrated by erythrocyte fragility tests and by changes in endpoint in complement and hemolysin titrations Collier et al[30] describe a method by which maximal packing is not necessary The true hematocrit value of an aliquot of washed erythrocyte suspension is determined by centrifuging it in a Bourke Ernstene hematocrit tube and from this value and that of the volume of the initial suspension the dilution of the original suspension required to

addition to the well known examples of mumps Newcastle disease and agents of the vaccinia variola group hemagglutinins have been found associated with a number of neurotropic agents such as the Columbia SK and Columbia MM viruses Theiler s encephalomyelitis virus members of the EMC group the Japanese B West Nile Russian Far East Western equine St. Louis and Murray Valley encephalitis viruses etc (See for example list in Casals and Brown)[37]

While the hemagglutination inhibition test theoretically is simpler to set up from the standpoint of the mechanics involved than in the complement fixation test this advantage is to a large extent nullified and even greatly overbalanced by a number of practical difficulties There is for example the matter of the agglutinability of the red cells Aside from differences in cells from different animal species even the commonly used chicken cells may differ from chicken to chicken Perhaps the extreme is seen in vaccinia where only one of numerous chickens may have suitable cells [38]

Another difficulty associated with the hemagglutination inhibition test is that it is relatively strain specific as is exemplified by influenza virus This characteristic is useful from certain research standpoints since it serves to reveal differences in the antigenic constitution of virus strains differences which are not revealed by the complement fixation test From the diagnostic standpoint however this is an undesirable characteristic since if the antigen used for antibody determination is markedly different from contemporary strains causing outbreaks detection of antibody rises may be missed In practical application this means that the antigen must be broad and also that in each outbreak or during each season prevalent strains should be isolated compared with the strains being currently used and if necessary replace the inadequate test strains In one recent outbreak of influenza B the sera of many patients showed a very low agglutination inhibiting antibody titer when measured by the Lee strain but a considerable titer and a diagnostically significant rise when measured by the Seattle strain Consequently diagnostic hemagglutination inhibition tests must be performed with the use of several strains of both the Type A and B viruses which is time-consuming It is simpler to do a single complement fixation test for Type A and Type B viruses which from the diagnostic standpoint is adequate especially since there is no specific therapy for this disease For typing of the outbreak hemagglutination inhibition tests are done with randomly selected specimens found positive by the complement fixation method

Of course one can use only a single strain each of the Type A and the Type B viruses in the hemagglutination inhibition method for diagnosis after the nature of the outbreak has been determined as to type While this may appear to be simpler than the use of the complement fixation test its execution is beset with other difficulties to wit the presence of non specific inhibitors in human sera which affect the reliability of the test [39,40]

chemical contamination Arsenicals alcohol anticoagulants such as citrate oxylate heparin preservatives and other substances may give rise to anti complementary properties in the serum The predominant cause of anticomplementary activity in our experience has been bacterial contamination of the specimen We do not use preservatives and handle all serums with sterile precautions at all times Simple sterility tests indicate that a high proportion of the serum specimens received contain contaminating microorganisms Such sera are filtered through Seitz pads and stored at 4° C which is our routine storage temperature In our opinion, storage of sera in the frozen state often deludes laboratory personnel into a false sense of security We have observed although we have no statistics as to incidence that sera which are repeatedly frozen and thawed quickly acquire anticomplementary activity Whether this is a function of the freezing and thawing per se cannot be stated since most of the anticomplementary specimens have been bacteriologically contaminated to some extent Such contamination results primarily from carelessness in handling the specimens under the supposition that good bacteriologic techniques and practice are not necessary when material is kept frozen

Everything considered the complement fixation method constitutes a good test Pains must be taken to titrate all the reagents as accurately as possible and to set the sensitivity at as high a level as is compatible with specificity The use of 50% endpoints is a refinement which is not required for diagnosis since a simple four fold rise in titer is sufficient on which to base a diagnosis Potent antigens however should be used insofar as possible since four fold rises may be of doubtful validity when the titers fall in the very low ranges for example a rise from 1 2 to 1 8 Recognizing that a high proportion of non specific reactions may occur at very low serum dilutions we have adopted a dilution of 1 8 as the initial one employed in the test As more highly purified antigens become available it may be possible to place some faith in these very low degrees of fixation but we do not consider it advisable to do so at the present time One virtue of the complement fixation technique is that it is but little hampered by the presence in the serum of non specific substances which give rise to so much difficulty with the hemagglutination inhibition technique Furthermore the complement fixation method is relatively insensitive to strain differences and for this reason alone it is in our opinion preferable to the hemagglutination inhibition technique for the diagnosis of such diseases as influenza While it is true that the existence of strain differences in some viruses has been suggested by the complement fixation test [35] differences in the sensitivity of the antigens produced from different strains must be adequately ruled out [36]

Hemagglutination Inhibition Tests Following the discovery that influenza virus possesses the capacity to agglutinate red blood cells a number of other viruses have been found to possess similar hemagglutinating properties In

what seems to be specific antibody may be misleading in the examination of specimens which contain non specific inhibitor [4]

As mentioned above hemagglutinins have been found associated with a number of neurotropic viruses also and hemagglutination inhibition tests have been devised for use with these agents In some instances the mechanics of performing the basic test appear to be as simple as those in the tests for influenza and mumps In other instances the procedures are rather complex as is indicated by the recent work of Sabin and his associates on viruses such as that of Japanese B encephalitis St Louis encephalitis and Western equine encephalomyelitis [49 50 1] The St Louis virus hemagglutinin is rather unstable and the comprehensive work of Chanock and Sabin [1] delineates the precautions which must be taken to insure preservation of the hemagglutinin Also serum contains a non specific inhibitor which is apparently associated with some lipid constituent inasmuch as it can be removed by extraction of the serum with acetone The diagnostic hemagglutination inhibition test as pointed out by Chanock and Sabin [1] is cumbersome and time consuming It is also intricate This adversely affects its adoption for large scale testing in a diagnostic laboratory and it is hoped that the procedure can be considerably simplified so that another tool may be added to the diagnostic armamentarium It would be valuable to know on the basis of a statistically significant number of observations how soon agglutination inhibiting antibody appears how rapidly the titer rises during the illness and how long it persists More detailed information is desirable as to the sensitivity of this method for antibody detection compared with other methods especially the complement fixation test

Agglutination Tests The biologically specific agglutination tests are feasible only with the larger viruses such as those of psittacosis and vaccinia and with the rickettsiae They are but little employed in diagnostic work for several reasons Comparatively pure suspensions of some of these agents are difficult to prepare but even where this is feasible the tests require rather large amounts of the antigen and the cost alone is sufficient to throw the balance in favor of the complement fixation method While it is true that microscopic agglutination tests either in capillary tubes or on slides have been described these generally meet with a cold reception not only because considerably more care must be exercised in executing these tests than is required for macroscopic methods but also because of the greater degree of error inherent in quantitative micro methods Macroscopic agglutination tests are sometimes useful in the rickettsioses for the examination of sera which are anticomplementary

Of the biologically non specific or heterogenic tests the Weil Felix method for the diagnosis of the typhus fevers is perhaps the best known This test constituted the only diagnostic method for many years until the specific rickettsial complement fixation tests became available about 1940 Since this latter test is biologically specific in the sense that a rickettsia and

Since the most detailed studies of inhibitors have been done with influenza virus (whose inhibitors are of the mucoprotein type) this agent may serve as an example Of the serum inhibitors active against this virus some are heat labile [4,41] as these are destroyed by heating at 56° for 30 minutes they are of no consequence in the test However at least two non specific inhibitors[4] which are heat stable are known and it is these that are of concern since they can profoundly affect the results of the test One of these the alpha inhibitor[4] is destroyed by the receptor destroying enzyme (*RDE*) present in *V cholerae* filtrates[43] and presumably is identical with the so-called Francis inhibitor [44] The other or beta inhibitor[4] is not affected by receptor destroying enzyme and is encountered to some extent in human sera although more commonly in animal sera

Non specific inhibitor may be present to such a degree that the true antibody titers may be obscured i e the inhibitor may prevent agglutination at much higher dilutions than does the specific antibody[4] Hilleman and Werner[45] found that non specific inhibitor of the acute phase blood often titrated higher than the specific antibody A true increase in antibody titer was thus masked and was not revealed until the paired serum specimens had been treated with RDE Since the non specific inhibitor in human sera is primarily of the alpha type most non specific inhibition can be removed by treatment with the *Vibrio cholerae* filtrate which is a tedious and time consuming step One recourse appears to be the replacement of inhibitor sensitive strains such as the PR8 FM–1 and Lee which are used in the standard Reference Test for Influenza Studies[46] with strains which are not sensitive to the inhibitor The FW–1 strain for example has been found[45] to be insensitive to alpha inhibitor and of additional importance it represents A prime strains of contemporary prevalence No strains insensitive to inhibitor have as yet been found among the Type B viruses

The problem of non specific inhibitors is not confined to influenza alone but can give rise to difficulties in other diseases where the hemagglutination inhibition technique is used as a diagnostic tool Thus on the basis of this technique the viruses of mumps and Newcastle disease were considered to be antigenically related whereas recent work has shown that this apparent relationship is spurious[3,33] It has been found that a non specific inhibitor is present in many sera obtained from apparently healthy humans[3] and that in addition sera containing specific antibodies against mumps virus also contain a heat stable heterotypic inhibitor substance for Newcastle disease virus[33] While cross reactions between the mumps and Newcastle disease viruses are observed in hemagglutination inhibition tests with considerable frequency similar cross reactions are only occasionally encountered in complement fixation tests[3]

It is also worthy of note that the concentration of non specific inhibitor in the serum of an individual may fluctuate considerably from time to time[47] and also that a four fold increase, i e a diagnostically significant rise in

these are not necessarily related to the simplicity of the procedure Thus the hemagglutination inhibition procedure per se is simple of execution but if it requires the use of a multiplicity of strains it becomes expensive from the standpoint of personnel time Similarly if in the case of influenza it is necessary to treat a proportion of specimens under examination with receptor destroying enzyme as part of the test the simplicity and the practicality of the method is adversely affected Likewise aside from the question of the role of the Coxsackie viruses in the causation of human illness it is hardly practical to conduct a battery of a dozen or more complement fixation tests for agents of this group

These points may seem so obvious that you may wonder why I have even troubled to mention them Yet they are frequently overlooked in the development of new diagnostic methods in virtually every field of microbiology and so lead me to stress the importance and the urgent need for the complete and thorough evaluation of any new diagnostic procedure Any test which is developed at the laboratory bench should go through repeated modifications until it is made as simple as is possible It should be given a thorough trial under both clinical and field conditions so that its sensitivity and specificity can be ascertained its practicality assessed and its usefulness in large scale examinations determined Only after such evaluation should the method be promulgated for routine application in a diagnostic laboratory One of the major factors leading to strained public relations between a diagnostic laboratory and its clientele is the premature promulgation for diagnostic purposes of methods which are too complex too time-consuming or otherwise impractical for application in routine work These difficulties are not engendered primarily by the investigators whose attitude generally is one of caution and restraint but by review articles and editorials in medical journals which fail to stress adequately if at all the intricacies and cumbersomeness of a method and tend virtually to emphasize that the method can be used to effect a diagnosis Several years ago when the Coxsackie viruses were in high fashion diagnostic laboratories were inundated with a flood of specimens for Coxsackie virus studies At the present time we are having similar difficulties with the poliomyelitis diagnostic problem and the situation is hardly improved by such well intentioned but over optimistic editorials as the one which appeared recently in a leading medical journal Those of you familiar with the current tissue culture techniques fully appreciate the stupendous task involved in testing hundreds of stool specimens for the presence of virus and its subsequent identification as to type and the examination of many hundreds of sera for rises in titer to each of the three known immunologic types of poliomyelitis virus I must emphasize also that the diagnostic laboratory is expected to conduct these examinations as the material is received so that the information is available on a current basis and not at some time in the distant future The laboratory is placed in an extremely difficult position when it

not the so-called X strains of *Proteus vulgaris* is the cause of spotted and typhus fevers its use in most laboratories has been preferred to the Weil Felix procedure There are many instances in which the results of the two tests are discrepant and oftentimes this can be traced to faulty technique in doing the Weil Felix test or to the use of improperly prepared antigen In addition the Weil Felix reaction is often positive in a number of conditions other than rickettsial infection A disturbing element has been introduced into the situation through the use of specific antibiotic therapy in the rickettsioses There have been a number of reports that antibody formation is interfered with to some extent during intensive antibiotic therapy and in at least one rickettsial disease viz Rocky Mountain spotted fever it has been suggested that complete suppression of specific complement fixing antibody may occur whereas the formation of X-agglutinins on which the Weil Felix reaction is based is but little if at all affected[5] Consequently, in some instances a diagnosis presumably is possible only through the use of the Weil Felix test

The cold agglutination test and the Streptococcus MG agglutination test are used in the diagnosis of primary atypical pneumonia since agglutinins to group O human erythrocytes or to the streptococcus appear in the blood of many patients during the course of this disease On the whole only about 50% of the patients[5] develop agglutinins for either of these antigens and hence a positive test constitutes supportive evidence for the diagnosis whereas a negative test does not rule out the disease It should be pointed out that the cold agglutination test is positive in a number of diseases and that this test as well as the Streptococcus MG test recently has been found positive in gastro-enteritis of children[63] the findings of these tests must be interpreted in the light of clinical and epidemiologic data

Comments

In this presentation the underlying thesis has been that no matter how well an immunologic procedure or an ancillary method may serve for the purposes of pure research or epidemiologic studies it does not necessarily follow that these same techniques or methods are suitable in routine diagnostic work as it applies to the everyday practice of medicine From the standpoint of the diagnostic laboratory an acceptable test must meet certain minimum criteria which may be listed as follows although not necessarily in order of their importance The test must be

1 Simple
2 Inexpensive
3 Practical
4 Sensitive
5 Specific

Specificity and sensitivity are prime requisites and an acceptable test must have a high degree of both As to the cost and the practicality of the method

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must refuse to do examinations because the cumbersomeness of a method multiplied by the factor of large volume places an impossible demand upon its facilities while the physician on the other hand is just as convinced from his reading that a diagnostic method is available once he has been inculcated with the idea that a diagnostic procedure has become available it is difficult indeed to convince him otherwise

In conclusion I would repeat that there is ample room for the simplification of basic techniques as applied to diagnostic virology Indeed the need for investigations on so seemingly prosaic a subject is sufficient to warrant at least some small part of the financial support accorded more esoteric inquiries

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Early Detection of Antigen as a Diagnostic Method

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The quest for diagnostic methods by which to identify an illness in its early stages has attracted the attention of many workers in many lands from the beginning of the microbiologic era to the present day As each new technic or instrument has been perfected it has been explored for potential usefulness in rapid diagnosis Ingenuity of a high degree has been apparent in some of the attempts but a review of the results shows a disappointingly short list of successes the failures on the other hand doubtless are far more numerous than the records indicate Various lines of approach to the problem of early diagnosis are dealt with in this symposium It is the purpose of this paper to evaluate as diagnostic methods the procedures for detection of antigen in viral and rickettsial diseases in the stage preceding the development of antibodies It is outside our scope to analyze the results of application of physical or physical chemical technics to the study of viruses and rickettsiae such as ultracentrifugation electrophoresis absorption spectroscopy electron microscopy amino acid analysis etc These methods require relatively large quantities of viruses furthermore the results are not highly characteristic to the point of providing positive identification without supporting evidence from immunologic or infectivity studies It is also outside our scope to discuss methods which involve propagation of a virus or a rickettsia prior to its identification Our attention focuses then on antigens and immunologic procedures

An antigen is usually defined as a complex substance the introduction of which into a foreign species is followed by the formation of antibodies specifically reactive with the antigen Even though it has been clearly established that intact rickettsiae and viral elementary bodies are antigenic in

our present discussion direct microscopic observations or electron micrographs of material from the diseased host are not pertinent unless combined with specific immunologic technics This eliminates the consideration of stained preparations of conjunctival scrapings in trachoma or inclusion blenorrhea of nodule contents in molluscum contagiosum of brain tissue in rabies of skin lesions in smallpox vaccinia chickenpox or herpes to cite a few examples

The paragraphs below mention very briefly the conventional immunologic reactions with a few illustrative examples where pertinent. The emphasis is then shifted to the discussion of the status of experimental methods which seem to be worth further study in the attempt to achieve early identification of antigens

Complement Fixation

For some systems satisfactory CF antigens are obtained from the tissues of experimentally infected animals and consequently the idea has been entertained by many workers that there might be enough antigen in materials obtainable from patients to fix complement in the presence of an excess of known antibody In the search for substances which can fix complement various antigens were discovered which were shown to be of smaller size than the infectious particle itself and often of distinctly different reactivity in the battery of immunologic tests Craigie [11] in 1932 discovered the soluble LS antigen of vaccinia Bedson and Lazarus and Meyer [3] described the antigens associated with psittacosis virus Smadel and Wall[39] reported the identification of soluble antigen in lymphocytic choriomeningitis Lennette and Horsfall [4] in 1940 published their studies of soluble antigens of influenza virus Topping[43] and Plotz [37] working independently found soluble antigens of epidemic typhus rickettsiae in 1942 Smadel Rights and Jackson[39] reported their demonstration of soluble scrub typhus antigens in 1946

To what extent have these complement fixing antigens been useful in actual diagnosis? The one good example is smallpox Satisfactory CF tests to identify antigen in materials from skin lesions were demonstrated by Gordon [19] Parker and Muckenfuss [3] Craigie and Wishart [12] and Downie [illegible] Furthermore specific CF antigen was detected in the blood of a severely ill smallpox patient before the eruption appeared [9] further successes in this regard were reported to the symposium by Downie[14] and by MacCallum [27] Their findings are of considerable interest and should stimulate renewed efforts in this direction

Except for smallpox the claims that diagnostic amounts of CF antigen have been demonstrated in the acute phase of viral and rickettsial diseases of man are viewed with varying degrees of scepticism To illustrate by one example Smorodintsev and Drobyshevskaya [41] and Leon [illegible] reported that serum from cases of typhus fever taken before the tenth day fixed complement in the presence of anti typhus serum These interesting papers were

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Early Detection of Antigen as a Diagnostic Method

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The quest for diagnostic methods by which to identify an illness in its early stages has attracted the attention of many workers in many lands from the beginning of the microbiologic era to the present day As each new technic or instrument has been perfected it has been explored for potential usefulness in rapid diagnosis Ingenuity of a high degree has been apparent in some of the attempts but a review of the results shows a disappointingly short list of successes the failures on the other hand doubtless are far more numerous than the records indicate Various lines of approach to the problem of early diagnosis are dealt with in this symposium It is the purpose of this paper to evaluate as diagnostic methods the procedures for detection of antigen in viral and rickettsial diseases in the stage preceding the development of antibodies It is outside our scope to analyze the results of application of physical or physical chemical technics to the study of viruses and rickettsiae such as ultracentrifugation electrophoresis absorption spectroscopy electron microscopy amino acid analysis etc These methods require relatively large quantities of viruses furthermore the results are not highly characteristic to the point of providing positive identification without supporting evidence from immunologic or infectivity studies It is also outside our scope to discuss methods which involve propagation of a virus or a rickettsia prior to its identification Our attention focuses then on antigens and immunologic procedures

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and protein content of the amniotic fluid caused confusion in interpretation of the curves Although this initial attempt was unsatisfactory I am none theless confident that the principle involved has merit and I am convinced that the technic should be investigated further preferably with more ver satile apparatus than the rather crude instrument I used

The electron microscope has been applied to observe the combination of viral particles with specific antisera by Anderson and Stanley[1] who noted apparent swelling of tobacco mosaic virus after contact with rabbit anti TMV serum Black Price and Wyckoff[3] published electron micrographs showing partially purified bushy stunt and southern bean mosaic viruses after exposure to control and to immune rabbit sera The viral particles in the immune serum were clumped and swollen by contrast to those in the control serum The quantities of plant viruses used for their tests were however tremendous compared with the amounts of animal viruses or rickettsiae which are ordinarily expected in infected tissue

Indirect Methods for Detection of Antigen Antibody Combination

Numerous tricks have been tried to render antigen antibody combinations visible including procedures like adsorption of one or both components on inert particles in suspension such as carmine indigo collodion bacterial cells or red blood cells A few of these tricks require comment for example the carmine particle technic of Smorodintzeff and Fradkina[40] as described for epidemic typhus Dr H R Morgan painstakingly and exhaustively ex amined the carmine particle method as well as several modifications only to conclude that its applicability was restricted solely to materials of high initial antigen content [31] More encouraging however was the report of O Connor and MacDonald[34] who applied the methods of Keogh et al [1 44] for coating erythrocytes with polysaccharide antigens to the detection of specific antigens in the urine of 13 cases of scrub typhus and 3 cases of murine typhus Their technic involves extraction of polysaccharide from proteus OX-K or OX-19 sensitizing erythrocytes with the antigens and determining the 50% endpoint of a human convalescent serum in titration with the sensitized cells Aliquots of the test serum at appropriate dilutions were mixed with sterile urine after 15 minutes sensitized red cells were added and the reaction was read in an hour The necessary types of controls were included A change of two fold or more from the expected titer of the known positive serum was taken as evidence of antigen in the urine This development certainly should be investigated further and in other systems to determine its practicability in diagnosis

Another phenomenon involving red cells was discovered by Dr Shih man Chang[6] in the rickettsial disease laboratory of the Harvard School of Public Health By heat and alkali treatment of *Rickettsia prowazeki* he obtained materials which render human group O red cells specifically agglutinable by typhus convalescent serum He named the material the erythrocyte sensi

promptly put to test by using specimens from the U S A Typhus Commission s library of sera from the severe epidemic in Cairo in 1944 Dr H R Morgan and I followed the directions of Smorodintsev meticulously but we obtained negative results [31] Later, a student at the Harvard School of Public Health Miss Harriet Boyd [4] adapted the spectrophotometric 50% hemolysis endpoint technic to our typhus studies she showed that her modification was 5 to 10 fold more sensitive than the conventional CF test for detection of antigen From her data and other experiments in our laboratory it is possible to calculate that the minimal amount of antigen detectable by CF test is roughly the equivalent of 10^5 typhus rickettsiae per ml Experience with other CF tests is in general agreement namely that relatively concentrated viral or rickettsial antigens are required To my knowledge the micro CF technic of Fulton and Dumbell[16] has not been applied to detection of antigen in human material

Combination of Antigen and Antibody

The end stage of the combination of antigen and antibody under optimum conditions is the development of precipitates or agglutinates which are visible by ordinary microscopy or by the naked eye For example in vaccinia the agglutination of elementary bodies was observed by Paschen[38] in 1913 Tanaka[4] noted flocculation in 1902 The applicability of this simple procedure is restricted to the very unusual circumstances in which a high concentration of antigen or elementary bodies is available

The stages preceding the formation of microscopic or visible aggregates in the combination of antigen and antibody molecules have not been adequately explored for possible application to early diagnosis I have reference in particular to the phenomenon of light scattering Inasmuch as the physical chemist can measure within a fairly wide range the size and even the shape of particles too small to resolve light by observing the intensity of light scattered at various angles from the incident beam[15] it seemed reasonable to consider the technic for study of antigen antibody reactions which do not proceed to the point of detection by ordinary means The method has been applied successfully in the study of the kinetics of relatively pure antigen antibody systems for example human sera vs its homologous horse antibody [17] crystalline bovine albumin vs partially purified anti bovine albumin rabbit serum [18] and a crystalline yeast dehydrogenase vs its homologous rabbit antibody [20] In my own experiments I tried to determine whether light scattering could be used to follow the events when infected fluids were brought into contact with a specific antisera for systems in which no changes were observed grossly or by microscopy In several trials with amniotic fluids from mumps infected embryos in contact with convalescent monkey serum changes in light scattering did in fact occur in the specific antigen antibody system but a satisfactory and rigorous control was not achieved for the reason that even slight variations in the amount of cellular debris

spread later to the interior of the nuclei where large accumulations finally were noted Watson[43] followed the progress of mumps virus infection of the chick embryo observing that the intracytoplasmic fluorescent granules were restricted to those cells which came into surface contact with infected fluid She further determined that the fluorescent staining of tissues closely paralleled the rise and fall in infectivity of the extraembryonic fluids

Dr Coons colleague Dr Chien Liu at the Harvard Medical School presented his fluorescent antibody studies of influenza A virus just a month ago at the Symposium on Fluorescence of the American Microscopic Society in Madison Wisconsin [6] He has generously allowed me to refer to his interesting observations which have not yet been published Dr Liu observed specific staining of the ciliated epithelium of the turbinates of ferrets undergoing infection by influenza A virus The specific staining increased in extent and the affected cells finally desquamated the fluorescence then was found in the cellular debris and in the nasal washings

Dr Liu was able to study 20 patients in a small epidemic of influenza A in Cambridge in 1953 He obtained nasal washings which were centrifuged lightly to sediment the cells for examination by the fluorescent antibody technic Virus isolation and hemagglutination inhibition antibody studies were undertaken concomitantly The agreement of the methods was definitely encouraging in this first application of the fluorescent antibody technic to clinical cases in an epidemic

Summary

The early detection of antigen as a diagnostic method has been achieved under certain circumstances for smallpox but in the case of the other animal viruses and the rickettsiae there are no simple reliable and practicable technics as yet Several interesting observations deserve further investigation particularly the fluorescent antibody method of Coons

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tizing substance or ESS At present the reaction is very useful for the study of antibodies in man but unfortunately ways to derive ESS from infected material of relatively low rickettsial content have not been devised and consequently no application of the phenomenon to the detection of antigens in human acute phase blood or urine has been made Its potentialities in diagnosis are being sought in various directions

Other lines of approach have been tried either with no success or with no increase in sensitivity over the CF test For example Makari [9] tested the Schultz Dale phenomenon with epidemic typhus materials Dr Shih man Chang tried to adapt to rickettsial studies the method of McMasters and Kruse[30] for the observation of vascular reactions in sensitized mice again without encouraging results [5] I shall not go into other unsuccessful experiments but rather devote the remainder of the time to studies made with labelled antibodies

Technics with Labelled Antibodies

Tracer technics are under investigation for the study of immunologic phenomena using I^{131} S^{35} azo-proteins etc The most promising technic from the viewpoint of detection of antigen is that developed by Dr Albert Coons of the Harvard Medical School This was referred to briefly by Dr Weller in his paper on tissue cultures [46] Dr Coons discovered that antibody molecules can be chemically conjugated with fluorescein without losing their specificity[8 9] and he has ingeniously devised technical methods for specific localization of several different antigens in thin sections or smears of animal or insect tissues The first application of his method to viral and rickettsial antigens was made by Coons Snyder Cheever and Murray[10] who reported the specific identification of epidemic typhus rickettsiae in cotton rat tissue and human body lice of Rocky Mountain spotted fever in cotton rat tissue and of mumps virus in monkey parotid gland

These results lead us to determine the *sensitivity* of the fluorescent antibody technic for detection of typhus rickettsiae and Dr F A Neva in the Harvard School of Public Health laboratory was able to show that here again as with the various other technics *before* regularly positive fluorescent preparations can be obtained there must be a concentration of rickettsiae well in excess of that which ordinarily occurs in human typhus materials [3] Drs Neva and Fagan then tried to identify rabies virus with the cooperation of Drs Cox and Koprowsky Their experiments with fixed virus and street virus both were unsuccessful they encountered non specific staining which was not eliminated by the absorption procedures found by Dr Coons to be adequate in other systems [33]

Drs Coons Coffin and Cabasso[7] have had excellent results in detecting canine hepatitis virus in the tissues of experimentally infected dogs The viral antigen was found in high concentration in the intranuclear inclusions the specific antigenic material first appeared on the nuclear membrane it

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DR J L MELNICK (Yale School of Medicine) I would like to pay tribute to Dr Weller s fine paper which has described the new techniques in his laboratory and which has certainly opened many new pathways to people working in virology One of the most exciting developments is the finding that in tissue culture there are certain viruses like varicella that will transfer from cell to cell only by contact infection of an infected cell with a neighboring susceptible cell So far this property seems to be associated with those viruses which produce intranuclear inclusion bodies At least we have come across another virus producing intranuclear inclusions which can propagate in this way in tissue culture

We recently isolated a strain of B virus from a monkey that was being used in a poliomyelitis experiment B virus is believed to be a common infection of monkeys just as herpes virus is a common infection of man Both viruses are antigenically related and both produce intranuclear inclusions Dr Frank Black has recently adapted this strain of B virus to epithelial cultures grown from monkey kidney tissue and the behavior of this virus in tissue culture is similar in certain respects to that observed by Dr Weller for varicella virus

The degeneration of tissue cultures caused by our local strain of B virus is distinctive both in the effect on individual cells and in the manner in which it spreads The first sign is the appearance of foci of infection (plaques) appearing on the sheet of epithelial cells For the first 48 hours the number of these plaques is proportional to the concentration of the inoculum Later many new plaques appear and degeneration soon becomes general The appearance of secondary plaques may be prevented by adding immune serum to the tube shortly after inoculating with the virus In this case the primary lesions spread gradually until the whole sheet is involved It thus seems that the micro-epidemiology of B virus infection is of a type intermediate between that of poliomyelitis and varicella B virus most readily infects in the manner of varicella by passing from one cell to another where they adjoin but it can also infect cells some distance from the first host by passing through the surrounding fluid as does poliomyelitis virus This character may account for the ability of members of the herpes simplex group of viruses to cause generalized infections in nonimmune persons and localized lesions in the presence of antibodies

To return to Dr Weller s varicella work it appears difficult or impossible to keep varicella or zoster virus going in serial passage unless he uses whole cells as passage material Yet the cultures can be started by the inoculation of cell free material from patients I would like to ask Dr Weller his interpretation of this apparent paradox

DR R THOMPSON (Indiana University Medical Center) I would like to ask Dr MacCallum about the capacity of variola virus to produce characteristic lesions on serial passage in the egg Also whether if one passes

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DISCUSSION

Laboratory Diagnosis of Virus and Rickettsial Infections

DR RHODES (Moderator) I am sure Dr Weller the applause will indicate to you how much we have enjoyed your talk It is so interesting to know this technique is not restricted to poliomyelitis virus but has a great many other applications Now we can afford about eight minutes for discussion Who would like to lead off? Everyone is very bashful this morning Maybe like Dr Meyer we are not sufficiently warmed up yet Well I would have one friend I can rely on Dr van Rooyen would you like to say something as to the diagnosis of smallpox with which you have had very extensive experience?

DR C E VAN ROOYEN (Connaught Medical Research Laboratories University of Toronto) The isolation of variola virus from the blood of human cases as early as the second and third day of illness during the pre-eruptive phase of the disease represents an important advance in the early diagnosis of smallpox The geographical location of the British Isles on the major lanes of air and sea communication make it imperative that laboratory diagnostic facilities for smallpox should be well organized and readily available for use in all suspected cases In this connection Dr MacCallum is to be congratulated on having effected the necessary administrative arrangements whereby ship s surgeons have been instructed to apply direct film scraping test to all skin eruptions of suspicious character

Although elementary bodies can usually be demonstrated in the early skin lesions of variola unfortunately in some 25% of cases of smallpox they are hard to find with the ordinary microscope Consequently the diagnosis remains in doubt

From a limited number of trials it would appear that better results and a higher percentage of positives may be expected by the use of the electron microscope Employing the latter variola elementary bodies appear as large brick shaped structures dense to the electron beam possessing a central area of electronic density and all of which features are easily recognized (See van Rooyen C E and Scott G D (1948) *Canadian Jl Pub Hlth* 39, 467)

without delay of the microbiological diagnostic procedures that will either provide a specific diagnosis or eliminate a variety of possibilities Certainly internists and pediatricians deserve the same help from microbiology that surgeons and gynecologists realize from the sister basic science pathology The patient benefits in hospital days saved by an adequate application of laboratory medicine The cost of seemingly extraordinary diagnostic aids is in line with ordinary laboratory media Each microbiologic diagnostic laboratory should have quickly available a variety of media for aerobic and anaerobic cultures as well as embryonated eggs 21-day-old and new born mice and finally in keeping with current developments at least the start of a tissue culture laboratory During the past two years the use of human and monkey cells in culture has opened up many possibilities for specific diagnosis For example a stable human cell strain HeLa Gey was reported last summer to support the growth of at least [illegible] viruses By having available known stock antisera for specific inhibition of the effects of each virus it is possible to apply this approach to the specific diagnosis of viral diseases with an accuracy and rapidity previously unknown To cite a problem that confronts physicians during the poliomyelitis season it now is possible to resolve the diagnosis for many cases with fever pleocytosis and little else For example poliomyelitis can be diagnosed by use of tissue culture techniques with result in the isolation and typing of virus in from 1 to 4 days The least time in our experience was 12 hours for an afebrile case admitted with involvement of the 6th and 7th nerves and a tentative diagnosis of brain tumor A specific diagnosis of poliomyelitis Type 1 was established over night The practical value of this approach and of similar methods in saving both hospital days and parents from worry that comes from uncertainty is obvious

In summary by applying laboratory diagnostic procedures available for viral diseases directly to the patient the student or house officer learns the value and the limitations of these diagnostic procedures how to collect specimens what specimens to use and which ones not to use Moreover the recognition by the physician that such facilities are available should discourage any immediate inundation of the patient with a variety of antibiotics and should lead the hospital staff and students alike to recognize that bacteriological studies are not to be accepted as negative from a single bacteriological culture Finally I do hope that this group as educators will accept the opportunities available for virus diagnosis and encourage the establishment of facilities in every modern hospital Retrospective antibody studies are not adequate Finally the problems that confront a viral diagnostic unit in a State Health Laboratory may not be comparable to problems in the hospital practice of medicine Even though both approaches demand trained personnel such as Dr Lennette has in his laboratory I believe it essential to work daily with the medical student and the house officer to acquaint them with the possibilities limitations and new developments in diagnostic virol

the virus repeatedly the rate of growth will increase or be more like that of vaccinia virus and too whether if one passes variola virus through the rabbit and returns it to the egg the lesions would be similar to those originally produced or more like that of the vaccinia virus

DR C E RICE (Department of Agriculture Hull Quebec) I will make my question very short I would like to ask Dr Meyer if he has any evidence of the development of non complement fixing and complement fixing antibodies in psittacosis and other viral infections and if there is any difference in the time of their development We now take it for granted in precipitation particularly in blood work that we may have both non precipitating and precipitating antibodies Our recent work on complement fixation indicates that we must also take into consideration the possibility that there may be a spectrum of antibodies which differ in their relative affinity for guinea pig complement

DR RHODES Our time is well in hand this morning We can allow about 20 minutes if necessary for discussion and then have the five speakers review very briefly their opinions about the various discussions

DR J T SYVERTON (University of Minnesota) Mr Chairman I should like to comment upon Doctor Lennette s summary of evaluation of virus and rickettsial diagnosis It is my understanding from Doctor Horsfall s remarks last night and from Doctor Lennette s summary today that both are in agreement that the laboratory diagnosis of viral diseases presents for other than research laboratories a completely impractical problem Doctor Lennette has cited his experiences as director of one of the most efficiently operated diagnostic laboratories in the United States That laboratory as a unit in a State Health Department gives physicians the privilege and they use it of submitting specimens in widest variety The problems that result may not apply to the application of viral diagnostic techniques in teaching hospitals and I qualify a teaching hospital as any unit with one or more internes or students Now to consider teaching hospitals where many of you have responsibility daily of inculcating students with basic principles by applying the case method of teaching The difficulties that result from a common failure in the daily practice of medicine to apply microbiological diagnostic methods because of the usage of multiple antibiotics are known to you That trend is difficult for the teaching staff in schools and hospitals to combat I believe that it would be a great mistake to overlook the opportunities for specific viral diagnosis currently open to personnel who are qualified in the virus and rickettsial field and who have direct access to patients for specimens and to an adequately equipped laboratory for carrying out basic procedures For example a patient with a clinical diagnosis of non bacterial pneumonia or of aseptic meningitis should be given benefit

without delay of the microbiological diagnostic procedures that will either provide a specific diagnosis or eliminate a variety of possibilities Certainly internists and pediatricians deserve the same help from microbiology that surgeons and gynecologists realize from the sister basic science pathology The patient benefits in hospital days saved by an adequate application of laboratory medicine The cost of seemingly extraordinary diagnostic aids is in line with ordinary laboratory media Each microbiologic diagnostic laboratory should have quickly available a variety of media for aerobic and anaerobic cultures as well as embryonated eggs 21-day-old and new born mice and finally in keeping with current developments at least the start of a tissue culture laboratory During the past two years the use of human and monkey cells in culture has opened up many possibilities for specific diagnosis For example a stable human cell strain HeLa Gey was reported last summer to support the growth of at least 8 viruses By having available known stock antisera for specific inhibition of the effects of each virus it is possible to apply this approach to the specific diagnosis of viral diseases with an accuracy and rapidity previously unknown To cite a problem that confronts physicians during the poliomyelitis season it now is possible to resolve the diagnosis for many cases with fever pleocytosis and little else For example poliomyelitis can be diagnosed by use of tissue culture techniques with result in the isolation and typing of virus in from 1 to 4 days The least time in our experience was 12 hours for an afebrile case admitted with involvement of the 6th and 7th nerves and a tentative diagnosis of brain tumor A specific diagnosis of poliomyelitis Type 1 was established over night The practical value of this approach and of similar methods in saving both hospital days and parents from worry that comes from uncertainty is obvious

In summary by applying laboratory diagnostic procedures available for viral diseases directly to the patient the student or house officer learns the value and the limitations of these diagnostic procedures how to collect specimens what specimens to use and which ones not to use Moreover the recognition by the physician that such facilities are available should discourage any immediate inundation of the patient with a variety of antibiotics and should lead the hospital staff and students alike to recognize that bacteriological studies are not to be accepted as negative from a single bacteriological culture Finally I do hope that this group as educators will accept the opportunities available for virus diagnosis and encourage the establishment of facilities in every modern hospital Retrospective antibody studies are not adequate Finally the problems that confront a viral diagnostic unit in a State Health Laboratory may not be comparable to problems in the hospital practice of medicine Even though both approaches demand trained personnel such as Dr Lennette has in his laboratory I believe it essential to work daily with the medical student and the house officer to acquaint them with the possibilities limitations and new developments in diagnostic virol

the virus repeatedly the rate of growth will increase or be more like that of vaccinia virus and too whether if one passes variola virus through the rabbit and returns it to the egg the lesions would be similar to those originally produced or more like that of the vaccinia virus

DR C E RICE (Department of Agriculture Hull Quebec) I will make my question very short I would like to ask Dr Meyer if he has any evidence of the development of non complement fixing and complement fixing antibodies in psittacosis and other viral infections and if there is any difference in the time of their development We now take it for granted in precipitation particularly in blood work that we may have both non precipitating and precipitating antibodies Our recent work on complement fixation indicates that we must also take into consideration the possibility that there may be a spectrum of antibodies which differ in their relative affinity for guinea pig complement

DR RHODES *Our time is well in hand this morning We can allow about* 20 minutes if necessary for discussion and then have the five speakers review very briefly their opinions about the various discussions

DR J T SYVERTON (University of Minnesota) Mr Chairman I should like to comment upon Doctor Lennette s summary of evaluation of virus and rickettsial diagnosis It is my understanding from Doctor Horsfall s remarks last night and from Doctor Lennette s summary today that both are in agreement that the laboratory diagnosis of viral diseases presents for other than research laboratories a completely impractical problem Doctor Lennette has cited his experiences as director of one of the most efficiently operated diagnostic laboratories in the United States That laboratory is a unit in a State Health Department gives physicians the privilege and they use it of submitting specimens in widest variety The problems that result may not apply to the application of viral diagnostic techniques in teaching hospitals and I qualify a teaching hospital as any unit with one or more internes or students Now to consider teaching hospitals where many of you have responsibility daily of inculcating students with basic principles by applying the case method of teaching The difficulties that result from a common failure in the daily practice of medicine to apply microbiological diagnostic methods because of the usage of multiple antibiotics are known to you That trend is difficult for the teaching staff in schools and hospitals to combat I believe that it would be a great mistake to overlook the opportunities for specific viral diagnosis currently open to personnel who are qualified in the virus and rickettsial field and who have direct access to patients for specimens and to an adequately equipped laboratory for carrying out basic procedures For example a patient with a clinical diagnosis of non bacterial pneumonia or of aseptic meningitis should be given benefit

making diagnostic tests on stool samples sent in from a few of the hospitals in Denmark and from Greenland The tissue culture technic is used for such tests As far as Coxsackie virus is concerned all stool samples received for virus studies are tested for the presence of Coxsackie virus by inoculation into newborn mice

For lymphocytic choriomeningitis we are using routinely the complement fixation test So far we have not found any positive reactions This is in accordance with the experience of laboratories in Sweden and Finland

A few sera have been tested for CF antibodies against the arthropod borne encephalitides These tests have all been negative

DR RHODES Thank you very much I think the remaining few minutes should be restricted to very short questions so that the gentlemen that have already spoken will have an opportunity to reply

DR F M HEYS (Baylor University School of Medicine) For about twelve years I have been supervising such a diagnostic laboratory for virus disease and to some extent for rickettsial disease first at the St Louis Children s Hospital (Washington University) with Dr Alexis Hartmann and Dr Russell Blattner and then with Dr Blattner at Baylor University College of Medicine in Houston where we have been in the process of setting up a diagnostic laboratory in connection with the Texas Medical Center development Of course in a Children s Unit the medical staff is able to control to some degree the type of patient selected for study However we are faced with the same problems which Dr Lennette and others have mentioned with pressure from the pediatrician and the practicing physician in the area who need aid in etiologic diagnosis This is especially true in Texas where laboratories equipped for this type of work may be rather widely separated During the twelve year period we have carried out studies more or less complete on about 400 spinal fluid specimens and corresponding blood specimens These patients were selected by the staff to be followed—usually for some particular reason Among them there have been about a dozen positives Seven were cases of lymphocytic choriomeningitis including one situation in which we were able to isolate the virus also from mice trapped in the homes of the patients and another situation in which the disease was occurring in an area of mill workers where mice were plentiful in the mill and in homes In two patients having a tentative diagnosis of post vaccinal encephalitis a strain of vaccinia was isolated from the spinal fluid in one of these viremia could not be demonstrated in either of two simultaneous blood samples Three of the patients in whom positive virus findings were obtained proved to have herpes simplex infections two of whom recovered one strain of herpes was isolated from spinal fluid during life and was verified by isolation of the same strain from autopsy material This particular work was done in collaboration with Dr James O'Leary and

ogy Certainly the outstanding hospitals such as the Henry Ford Hospital can serve by applying every facility currently available for the laboratory diagnosis of viral infections

DR RHODES Thank you very much Dr Syverton Ontario might warmly reciprocate in that they have a very large number of people here I am going to exercise the Chairman s prerogative of calling on certain representatives from European countries We so seldom have the opportunity of hearing them I know for example that Dr Sven Gard from Stockholm is interested in this problem I wonder if I might ask him to say a few words

PROF S GARD (Caroline Institute Stockholm Sweden) To tell the truth I hesitate quite a lot to enter into this discussion I am in a position more or less of the type that Dr Syverton outlined I too have to teach students and I think that much of what Dr Syverton said has to be considered very carefully I think personally that the value of virus diagnosis will grow rapidly in clinical work and particularly perhaps in the field of Public Health I feel that we should try to encourage the creation of as many virus laboratories as possible at different capacity levels I would advocate a system of regional laboratories with limited facilities but nevertheless able to take over some of the necessary tests—let s say the complement fixation tests—and also able to collect the proper material It is of particular importance to have regional laboratories in contact with hospitals and patients they should see to it that the samples are collected in the proper way and if necessary further delivered to central or reference laboratories better equipped to deal with the more delicate and more complicated methods I think that without trying—without really looking for something—we will never find anything I am of the same opinion as Dr Syverton that no matter what the cost will be and knowing that at the present stage certainly much labor and money will be wasted on work that doesn t lead to any immediate results—no matter what the costs are the work should be done and eventually we will be able to develop more effective methods and see our aims more clearly Thank you

DR RHODES Dr Herdis von Magnus would you like to make any comments on that situation in Denmark? She is part of the scientific family here and I would like to know if Mrs von Magnus is going to make any remarks Would you like to make a few remarks as regards routine diagnosis in Denmark?

DR HERDIS VON MAGNUS (State Serum Institute Copenhagen Denmark) In our institute the diagnosis of influenza and mumps viruses is made routinely on request from the hospitals Complement fixation tests are performed usually on paired serum samples As regards polio we are by now

the blood which specifically precipitated with antirickettsial serum Have you ever attempted to demonstrate antigen in the clot rather than in the serum with your techniques?

DR ALICE MOORE (Sloan Kettering Institute for Cancer Research New York City) I think we ve had rather a unique experience and want to commiserate with Dr Lennette Dr Southam and I have inoculated around two hundred far advanced cancer patients with three different types of neuro-encephalitic viruses namely West Nile Ilheus and Bunyamwera and have followed the patients very carefully I must say that if we didn t know what we had given them we certainly would have had a hard time making a diagnosis Most of these patients do not get any symptoms whatsoever They often have a viremia the degree depending on the virus and also depending on the individual for perhaps a week The longest viremia we have had has been about three weeks But you would scarcely suspect that because most of these patients are afebrile We have had some signs of encephalitis and also have had some severe encephalidites but at the time of the encephalitis not only are the bloods negative but also spinal fluid examination usually shows no virus We have retrieved some virus on occasion but the great percentage of them are negative so it is quite a problem to be able to diagnose these encephalidites

DR RHODES Any more questions? We still have a few minutes

DR VAN ROOYEN I likewise have had difficulty in being able to repeat the indigo carmine test described by Dr Smorodinseff and others

DR SABIN The purpose of this conference is to find out what we can do—what we have learned—to make it possible to diagnose virus diseases earlier or as early as possible and we are spending all the time discussing administrative problems I think it was all right at dinner last night but it is not fair to the fine papers and it does not make a scientific meeting out of this

DR G A LOGRIPPO (Henry Ford Hospital) I agree with Dr Sabin Our problem is to try to extract the antigen from the patient when he is sick We know the antigen is there but we have not been able to extract it in sufficient quantities with our present methods However with the ion exchange resins this is now possible At least it is possible to extract polio virus and coxsackie virus from human feces This is a new tool in virus work and I am not prepared to say how fruitful it is going to be for early diagnosis but I would like to mention it at this time I have demonstrated the use of the ion exchange resins in partial purification and concentration of polio virus from CNS tissue with a certain combination of resins Now

Dr Margaret Smith in St Louis In one instance the virus of St Louis encephalitis was isolated from the blood of a child who showed minimal clinical signs

In patients presenting cutaneous manifestations we have had some quite satisfactory results with a fair degree of success in establishing etiologic diagnosis On several occasions the problem of differential diagnosis arose in patients showing skin rash for example two cases of meningococcemia occurred in children residing in an area where Rocky Mt Spotted Fever was endemic and from which several patients with this disease had been admitted This work has been facilitated greatly by the use of the embryonated egg as a supplement to animal inoculation and the usual bacteriologic culture methods We are hoping to have tissue culture methods available in the near future

DR MERRILL J SNYDER There is something that I believe these gentlemen have overlooked Those workers in combined routine and research viral laboratories have particular research interests Their research specialties are known to all of us through their publications In our diagnostic laboratory we are equipped because of personnel and other limitations only to perform the simpler procedures For example when spinal fluid is received from a case of serous meningitis mice and guinea pigs are inoculated intracerebrally If the agent happens to be lymphocytic choriomeningitis virus it is a simple procedure to make some splenic antigen from the infected guinea pigs and identify the virus by complement fixation However if another virus is involved and only the mice sicken neutralization and cross immunity tests for the final characterization of the virus are too complex and time consuming to carry out in a laboratory such as ours There are those engaged in research with the neurotropic viruses who I am sure would be most pleased to receive this potentially fruitful material If one felt that he had a possible isolation of Q fever in guinea pigs I feel certain that Dr Lennette would be happy to receive material from the case It is those of us in the small diagnostic laboratories rather than the workers in the large centralized ones who see the patients and are in direct contact with the physicians The taking of proper specimens can therefore be assured and the first screening procedures can be performed promptly It is in this way that knowledge of new viral diseases and the understanding of the pathogenesis of viral infections will have the best opportunity for progress

I should like to also ask a question of Dr Snyder Dr Snyder I am reminded of two things One is the clot procedure for the isolation of rickettsiae which many have claimed is more successful than isolation from whole blood The other is the Japanese method for the early serological demonstration of typhus antibodies in blood It was claimed that with their method of bile digestion and acid precipitation of blood clot taken during the first two days of disease they were able to extract and concentrate antigen from

DR MACCALLUM There were just one or two questions which perhaps I should try and answer without discussing any of Dr Lennette s and my own difficulties Dr van Rooyen the question of electronmicroscopy I think I would agree with Dr Lennette s remarks We have not got an electronmicroscope We don t think it is of sufficient practical value at this stage to put its price against all the other things we could buy for the same amount of money but if you ve got one then of course one can test its value in diagnosis of pox infections But the difference between the appearance of varicella and the other pox viruses I should think is so slight that I would still like to rely on my biological test for the differentiation But that is only the present position which may alter As to Dr Thompson s question about changes in the macroscopic appearance of the pocks from variola on continued passage I think the answer is that they don t change Some years ago Dr Nelson at the Rockefeller Institute passed variola and vaccinia together on chorio allantoic membrane for some 25 passages and they each retained their specific appearance The question of what happens to variola when you pass it through a rabbit and then back to the mem brane I think is one of those questions that needs further investigation and just to satisfy myself at this present time I am investigating the old question of How can you make vaccinia from variola? We have reached the stage of passing fresh virus from man through the monkey and into the rabbit and so far it has not changed its appearance after some several passages through the rabbit There is one other test which is pertinent to this dis cussion today although influenza hasn t been mentioned and it goes right back to the first paper of the meeting by Dr Gottschalk and that is the Fazekas test for detection of inhibitor in nasal mucous at the onset of in fection with influenza Fazekas in Melbourne has described this test and said that he has obtained good correlation with it and his virus isolations One of the members of my staff has been working with this and it certainly is not as simple as it appeared from reading Fazekas papers We still can t say whether this has any practical application but it may be a useful method of obtaining an early diagnosis of influenza and doing away with the use of eggs and perhaps doing away with some of the bleeding Just one gen eral point which is slightly off the question of early diagnosis but which I think it is time we started to think about and that is the question of some standard sera so that we each know what the other man is talking about when we are given results on complement fixation tests We are in a fortunate position in that the virus diagnostic tests for lab service have been developed first in the central laboratory in England We make our own antigens and control sera There are now ten Public Health Labs in the country doing these tests and for all of our associates doing this work we provide the antigens the control sera and the technique so we are all using the same methods Our C F tests are all done in plastic plates with cups as used for haemagglutination and this has done away with the

it is also possible to extract the polio virus and the coxsackie virus from human feces with a different combination of resins This is a very simple procedure and it may fit in with Dr Lennette s request for procedures necessary for the routine laboratory I have used as little as 1 liter and as much as 10 gallons of a 1% fecal suspension and extracted the virus in several hours or over night depending upon the volume processed I think if we can continue in this trend we might be able to work on some of these problems and develop a procedure for the hospital laboratory The crude fecal suspension is added to a column of ion exchange resins and passed through over night You have your virus antigen in the effluent which is collected in another container in which a second resin has been placed, the second resin absorbing the bulk of the protein material and the virus Next morning you can reduce this volume from 10 gallons down to about 1 liter If we can get an antigen out of a mouse there is no reason why we can t get the virus antigen out of man especially when he is excreting it The difficulty with the poliomyelitis virus has been that it is not a complement fixing antigen whereas the coxsackie virus is The coxsackie virus antigen was found to be anticomplementary and this was due to small traces of the phosphate which can be eliminated by dialysis Now it remains to develop some method with which to detect the antigen that has been extracted If use is made of the ion exchange resins in trying to extract these antigens from man during the acute phase of illness perhaps serological methods can be found to detect unknown virus antigen with known sera These resins for this entire procedure cost about $2 60 and ordinary laboratory equipment will do the job

DR PUCK I agree with the point of view expressed that the use of ion exchange resins in virology offers a great many promising applications Its use in virus purification and concentration was first demonstrated by LoGrippo using anionic exchangers and by our laboratory among others with cationic exchangers Under certain conditions these reagents can also be made to split phage into its protein and DNA components Finally a recent application affords promise in isolating and concentrating antibodies a procedure which might prove of value in diagnostic procedures Dr Lerman at our school has done the following experiment He has coupled to an ion exchange resin the specific antigen in which he was interested and then simply poured serum containing antibodies to this antigen through the column As could be expected the specific antibody remained behind coupled to its antigen and all the rest of the protein and other serum components were thus removed The column was then washed thoroughly after which he was able to re elute the antibody obtaining considerable purification and concentration Ion exchange procedures are extremely simple and require a very minimum of apparatus I believe the technique offers an exceedingly promising tool for virology

DR MACCALLUM There were just one or two questions which perhaps I should try and answer without discussing any of Dr Lennette's and my own difficulties Dr van Rooyen the question of electronmicroscopy I think I would agree with Dr Lennette's remarks We have not got an electronmicroscope We don't think it is of sufficient practical value at this stage to put its price against all the other things we could buy for the same amount of money but if you've got one then of course one can test its value in diagnosis of pox infections But the difference between the appearance of varicella and the other pox viruses I should think is so slight that I would still like to rely on my biological test for the differentiation But that is only the present position which may alter As to Dr Thompson's question about changes in the macroscopic appearance of the pocks from variola on continued passage I think the answer is that they don't change Some years ago Dr Nelson at the Rockefeller Institute passed variola and vaccinia together on chorio allantoic membrane for some 25 passages and they each retained their specific appearance The question of what happens to variola when you pass it through a rabbit and then back to the membrane I think is one of those questions that needs further investigation and just to satisfy myself at this present time I am investigating the old question of How can you make vaccinia from variola? We have reached the stage of passing fresh virus from man through the monkey and into the rabbit and so far it has not changed its appearance after some several passages through the rabbit There is one other test which is pertinent to this discussion today although influenza hasn't been mentioned and it goes right back to the first paper of the meeting by Dr Gottschalk and that is the Fazekas test for detection of inhibitor in nasal mucous at the onset of infection with influenza Fazekas in Melbourne has described this test and said that he has obtained good correlation with it and his virus isolations One of the members of my staff has been working with this and it certainly is not as simple as it appeared from reading Fazekas' papers We still can't say whether this has any practical application but it may be a useful method of obtaining an early diagnosis of influenza and doing away with the use of eggs and perhaps doing away with some of the bleeding Just one general point which is slightly off the question of early diagnosis but which I think it is time we started to think about and that is the question of some standard sera so that we each know what the other man is talking about when we are given results on complement fixation tests We are in a fortunate position in that the virus diagnostic tests for lab service have been developed first in the central laboratory in England We make our own antigens and control sera There are now ten Public Health Labs in the country doing these tests and for all of our associates doing this work we provide the antigens the control sera and the technique so we are all using the same methods Our C F tests are all done in plastic plates with cups as used for haemagglutination and this has done away with the

cleaning of very large numbers of tubes We also use the Fulton Dumbell technique on flat plastic plates for studies on human sera but still only to a limited extent because we haven't enough boxes necessary to keep the plates in the hot room and cold room to prevent evaporation We use these other plates with 0 1 ml volumes and on the whole we use short fixation for all tests except variola and L C M where we find that overnight fixation is necessary to detect smallest amounts of antibody We only use 1½ hours fixation so that the plates can just be stacked one on top of another in boxes in the hot room then in a waterbath ½ hour and the whole test is through in 2 hours

DR WELLER *Dr Melnick has a specific question regarding the status of* the agent of varicella in tissue culture as compared to that present in vesicle fluid Obviously there is a very basic and important difference We are only starting to study this agent in tissue culture and I think if we call it virus now it will be a slip of the tongue We do not yet have conclusive proof that we are working with a virus for the agent remains associated with the tissue phase of the culture system and filtration experiments have to date been unsuccessful Otherwise I am only too happy that Dr Snyder had a chance to give his presentation of Dr Coons technique The fluores cent *antibody technique promises to have wide applications in the field of* tissue culture work as related to the problems of the diagnosis of viral infections

DR LENNETTE I do not know how to separate the numerous questions which were raised and so I shall try to answer all the pertinent ones in the form of a brief discussion First of all I am sorry Doctor Sabin if I ap peared to be unduly pessimistic here and last night I did not intend to leave an air of gloom since I am highly pleased with the advances that have already been made in the *routine* diagnosis of viral and rickettsial disease and I am most optimistic that the future will bring us additional techniques more simplified methods and also means for the diagnosis of infections not currently amenable to laboratory diagnosis What I actually undertook was a plea for more realism in dealing with what is truly *applied* diagnosis that *encompassed by the general term "diagnosis" All of us are aware* that each year cures for this or that are announced giving rise in the public mind to false hopes which are eventually dashed On the medical side I think we sometimes inadvertently give rise to a similar situation insofar as the clinician is concerned We talk about new diagnostic tools which have been developed we promulgate their use and give rise *to the impres* sion that here is a new method which is immediately available Basically this may be true but since the same word may mean different things to different people the matter resolves itself into how one interprets the term "diagnosis" Thus in such situations as that described by Doctor Syverton

for example when one is dealing with a single institution one can select the patients on whom a thorough laboratory investigation is desirable One has a full case history a good clinical background and an epidemiological background all of which are invaluable Such a situation is ideal but is encountered usually in university hospitals or associated institutions

Then there is that situation in which a diagnosis is conducted by an investigator or an investigative team Let us take poliomyelitis as an example In a laboratory whose primary or sole concern is the study of poliomyelitis attempts at laboratory diagnosis of this disease bring to bear the full resources of that laboratory which is concerned with that disease to the exclusion of others The same might be said of influenza or mumps or typhus fever

In the first situation the work load is comparatively small In the second situation personnel time money and cumbersomeness or unwieldiness of a methodology are not necessarily prime considerations Compare these situations to that of the reference diagnostic laboratory which deals not with one disease but with a wide variety of viral disease occurring within its area of operation and with a large amount of material submitted daily In the case of a state virus reference laboratory such as ours we deal not with a few hundred specimens but with thousands not with single tests but with a battery of tests (48 000 tests during 1952) and not with the staff of a single hospital or a small group of hospitals but in theory at least with virtually every practicing physician and health officer within the state Material sent in for examination is just that—complete clinical histories are unusual there is no epidemiological information and the examinations must be done rapidly and expeditiously The gist of my plea and the underlying thesis of my paper is to point out that there exists a wide chasm between what a research or investigative laboratory may consider a diagnostic procedure and what a reference laboratory doing a huge volume of work considers feasible Because of current interest let us use poliomyelitis as an example A poliomyelitis research laboratory testing several hundred stools yearly may consider tissue culture methods a diagnostic tool Would it do so if it had to examine several thousand stools yearly on a *current* basis? If it undertook such a task I am sure it would conclude as we have that the method is not practical for *large scale* examinations I have tried to point out some of the shortcomings of our present methods and the great need for their simplification The need for investigation into such prosaic subjects as techniques and methods was well impressed upon us in the summer of 1952 for example when there occurred in California over 1000 cases of central nervous system disease most of which was considered on clinical grounds to be encephalitis Out of this group we were able to show that several hundred cases were due to Western equine encephalitis virus The diagnosis was effected on the basis of the complement fixation method When however the neutralization test was used we were unable in an

amazingly large proportion of these cases to show that there was a diagnostically significant rise in neutralizing antibody titer this difficulty arose apparently because the neutralizing antibody had already reached its maximum level by the time the first blood specimen was obtained The usual type of neutralization test i e one in which constant serum varying virus dilutions are used was employed This raises the question as to whether this problem might not be resolved by the use of a test employing constant virus varying serum dilutions The work by Tyrrell and Horsfall and his collaborators points to the value of such a method Certainly there is ample work to be done in the simplification of our methodologies and it is to be hoped that investigators will devote some of their time to the improvement and refinement of the basic tools used in research and that support for such work might be made available

DR JOHN C SNYDER Two questions were asked (1) Have we tried the clot method for detecting the viable rickettsiae? Yes we have given that an exhaustive trial (2) Have we tried the method reported by Japanese workers based on the detection of antigens in patients blood? Their report indicated that positives occurred about the 15th day of the disease We did not get positive results on specimens from Cairo taken on the 3rd day of illness by using their method as reported

Part V

Approaches to Prophylaxis and Therapy of Virus and Rickettsial Infections

Moderator

Gilbert Dalldorf

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Interference and Physical chemical Blockade

Frank L Horsfall, Jr
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A rational approach to the prophylaxis and therapy of virus diseases by chemical means might be made if the biosynthetic processes involved in virus reproduction or in the metabolic aberrations caused by the presence of viruses were definable in chemical terms It is clear from results reported earlier in this Symposium that although new information is accumulating the biochemistry of virus multiplication and of the damaging effects caused by viruses are still obscure In the absence of adequate information about these phenomena most approaches to the problems mentioned have of necessity been empirical In spite of this some solid data have been obtained and of more importance means for securing further information are becoming apparent

In the animal virus field two well known phenomena bear on the subject under discussion In operational terms these are (1) Host tissue infected with one virus may not be infectable by another virus (2) infected host tissue may yield less virus when certain chemical substances are added The first phenomenon is termed virus interference the second is designated chemical inhibition of virus multiplication There are indications that both phenomena can be attributed to alterations in the biosynthetic mechanisms necessary for virus reproduction Data on the multiplication of viruses becomes more secure the more closely they can be related to the physical units in the system the individual cells and virus particles With animal viruses techniques only recently have become available for a direct approach to these primary units The work of Dulbecco[1] with plaque forming animal viruses has raised the possibility that an approach at the individual cell level can be made In addition our own work and that of Levine et al [3] indicate that precise enumeration of hemagglutinating virus particles by photometric means is feasible Much of the published data on virus

interference and chemical inhibition of virus multiplication have been secured in studies on whole tissues or intact animals inoculated with small amounts of virus Recently more quantitative work has been done by kinetic studies during single cycles of multiplication

When influenza virus is injected into the allantoic fluid of an embryonated egg the allantoic membrane becomes incapable of supporting the reproduction of a different influenza virus [4,5] As Forssman[6] has shown recently in our laboratory insusceptibility to the challenge virus regardless of the quantity injected is present at two hours though not at one hour after injection of the interfering virus Such a result is obtained when the number of the first or interfering virus particles is about five times the number of allantoic cells When smaller numbers of virus particles are inoculated a longer time is required before insusceptibility to the challenge virus develops [4] The length of the interval is in inverse relation to the number of interfering particles inoculated and the data are in accord with the view that tissue insusceptibility appears only when all or nearly all cells are infected The insusceptibility cannot be explained on the basis of blockade at the cell surface for the challenge virus is adsorbed by infected tissue in normal manner [7] Whether the second or challenge virus or some part of it goes on to penetrate the insusceptible cell is not yet known The data indicate that insusceptibility develops during the latent or plateau period of the interfering virus considerably before its progeny can be found Thus it appears that the first virus to reach the cell initiates events in the course of the reproductive process which produce insusceptibility to the challenge virus

Whatever the nature of such changes in the infected cell may be they are clearly not dependent on the integrity of the entire reproductive process Heterologous inactivated virus which does not reproduce also leads to insusceptibility of the cell whether inactivation is caused by ultraviolet irradiation [5,8] heat [9] or spontaneous decay [5,8] As would be expected inactivated virus does not produce insusceptibility in the allantoic membrane unless much is injected [8,10] about 5 inactivated particles per cell appear to be necessary [6] This is in agreement with the data for heterologous infective particles and *supports the idea that it is necessary to block all or nearly all* cells before insusceptibility appears

Inactivated particles also can block the reproduction of infective particles of the same or homologous strain [5,8] This has been designated auto interference The means by which inactivation is obtained is again not critical and ultraviolet irradiation [5,8] heat [11] or spontaneous decay [5,8] yields preparations which cause auto-interference Evidence of interference is obtained even when heat inactivated and infective particles of the same strain are injected simultaneously if the number of inactivated particles is sufficiently large [5,8] *i e* about 5 per allantoic cell [12] With smaller inactivated particle cell ratios the yield of virus from the infected tissue is diminished to an extent which corresponds with computations on the proportion of cells

blocked [12] Such computations are based on the assumption that the blocking reaction is all or none and individual cells are either blocked or not blocked by inactivated virus particles An alternative assumption is that inactivated particles cause a reduction in the yield from all cells in direct relation to their concentration The available data do not permit a decision between these two possibilities

Both infective and inactivated influenza viruses cause host tissue to become insusceptible to infection with the same as well as certain unrelated viruses The blocking effect is rapidly induced during the latent period of infective particles but is not persistent and even in the mammal lasts no more than a week or two [13] The alteration has specific features and does not lead to insusceptibility to all viruses capable of infecting the host tissue Thus either mumps or pneumonia virus of mice can reproduce concurrently with influenza viruses in the same tissue [7] Whether such noninterfering agents actually multiply side by side in the same cells is not yet certain There appears to be no way to predict if two viruses will show interference or whether it will be reciprocal Immunological relatedness similarity in size or capacity to multiply in the same tissue is not a necessary character in pairs of agents which show interference Thus influenza virus blocks infection by Western equine virus in the mouse brain [13] and mumps blocks reproduction of PVM in the mouse lung [14] though neither of the first or interfering viruses reproduces in the classic sense in the host tissue used

There appears to be no instance in which it has been established that reproduction of the first virus to reach the cell is blocked within the cell by the later introduction of a second virus The interference phenomenon provides a means for preventing the reproduction of a virus but gives no indication that it can lead to the interruption of reproduction once the process has been initiated Reports of so-called reverse interference [8 10] in which the second or challenge virus appeared to modify the course of infection by the first can be explained readily The small inocula used result in infection by the virus of only a small proportion of cells and the later injection of a large quantity of the second virus results in interference with reproduction in the remaining cells

With the preparations of influenza virus that are generally employed the injection of very large amounts of the agent into the allantoic cavity leads to slower rates of appearance and smaller yields of virus than are secured when small amounts are inoculated Moreover as von Magnus[15] has shown a large proportion of virus particles in the yield are non infective Our own experiments with precise enumeration procedures reveal that under such circumstances the ratio of infective to hemagglutinating virus particles in the yield may be as low as 0 01 or less

The infectivity of influenza virus particles is a very unstable property In allantoic fluid at 35 C either *in vitro* or in the egg the half life of infective particles is about 150 minutes [12] Unless extraordinary precautions

interference and chemical inhibition of virus multiplication have been secured in studies on whole tissues or intact animals inoculated with small amounts of virus Recently more quantitative work has been done by kinetic studies during single cycles of multiplication

When influenza virus is injected into the allantoic fluid of an embryonated egg the allantoic membrane becomes incapable of supporting the reproduction of a different influenza virus [4] As Forssman[6] has shown recently in our laboratory insusceptibility to the challenge virus regardless of the quantity injected is present at two hours though not at one hour after injection of the interfering virus Such a result is obtained when the number of the first or interfering virus particles is about five times the number of allantoic cells When smaller numbers of virus particles are inoculated a longer time is required before insusceptibility to the challenge virus develops [4] The length of the interval is in inverse relation to the number of interfering particles inoculated and the data are in accord with the view that tissue insusceptibility appears only when all or nearly all cells are infected The insusceptibility cannot be explained on the basis of blockade at the cell surface for the challenge virus is adsorbed by infected tissue in normal manner [7] Whether the second or challenge virus or some part of it goes on to penetrate the insusceptible cell is not yet known The data indicate that insusceptibility develops during the latent or plateau period of the interfering virus considerably before its progeny can be found Thus it appears that the first virus to reach the cell initiates events in the course of the reproductive process which produce insusceptibility to the challenge virus

Whatever the nature of such changes in the infected cell may be they are clearly not dependent on the integrity of the entire reproductive process Heterologous inactivated virus which does not reproduce also leads to insusceptibility of the cell whether inactivation is caused by ultraviolet irradiation [5 8] heat [9] or spontaneous decay [5 8] As would be expected inactivated virus does not produce insusceptibility in the allantoic membrane unless much is injected [8 10] about 5 inactivated particles per cell appear to be necessary [6] This is in agreement with the data for heterologous infective particles and supports the idea that it is necessary to block all or nearly all cells before insusceptibility appears

Inactivated particles also can block the reproduction of infective particles of the same or homologous strain [5 8] This has been designated auto interference The means by which inactivation is obtained is again not critical and ultraviolet irradiation [5 8] heat [11] or spontaneous decay[5 8] yields preparations which cause auto-interference Evidence of interference is obtained even when heat inactivated and infective particles of the same strain are injected simultaneously if the number of inactivated particles is sufficiently large [5 8] *i e* about 5 per allantoic cell [2] With smaller inactivated particle cell ratios the yield of virus from the infected tissue is diminished to an extent which corresponds with computations on the proportion of cells

inhibits the reproduction of infective particles Inhibition is obtained with either compound even though the substance is not added until half of the latent period has passed Proflavine inhibition especially warrants comment because of recent findings On removal of this compound inhibition disappears and infective phage particles are produced promptly In the presence of the compound as Foster showed[17] lysis of the bacterial host cell occurs at the expected time even though no infective virus is released This surprising result was explained by De Mars *et al*[18] Proflavine blocks a late stage in the reproduction of phage and only tailless particles—so-called doughnuts—are produced and released on lysis of the infected bacterium Such tailless phage particles are non infective and do not register in plaque counts but can be found with the electron microscope

Although the biochemical basis for the inhibiting effect of proflavine has not been defined certain deductions can be made as to the mode of action Blockade of adsorption cannot be implicated for the effect is obtained long after adsorption is completed In contrast to blockade of reproduction by means of virus interference the compound interrupts virus reproduction in cells already infected and is operative only during a relatively late stage of the latent period[17] That reproduction itself is affected is evident from the fact that tailless or incomplete particles are formed and are not identical chemically or immunologically with mature infective phage particles[18] Despite this profound effect on the reproduction of the virus the host cell is damaged and reacts in the same way it would if phage multiplication had proceeded normally It bursts open and dies at the expected time Thus in this case chemical inhibition of virus reproduction does not alter the outcome of the infection as regards the bacterial host cell

The reproduction of some medium or small size animal viruses can also be affected by the addition of chemical compounds during the latent period To demonstrate this with these agents is technically more difficult and less unequivocal than with bacterial viruses but can be achieved if the single multiplication cycle procedure and quantitative methods are employed

The multiplication of pneumonia virus of mice[19] influenza[20] and mumps viruses[21] can be inhibited during the latent or plateau period after infection of the cell is in progress These agents have diameters of about 40 100 and 130 millimicrons respectively With each virus[1] [3] inoculation of a very large number of particles leads to events which are closely similar to those obtained in one step growth experiments with bacterial viruses With influenza viruses computations indicate that the number of infective particles injected is sufficient to produce nearly simultaneous infection of all the allantoic cells[1] With the other two agents the number of virus particles used is large enough to infect a high proportion of available cells Under these circumstances kinetic studies give an indication of developments at the cell level

Highly purified capsular polysaccharides of *K pneumoniae* inhibit the

are taken and fully infective virus is harvested immediately after release into the allantoic fluid most preparations contain 90% or more non infective virus particles. When injected in large numbers such spontaneously inactivated particles act in the same manner as ultraviolet or heat inactivated particles and cause a large proportion of the allantoic cells to become insusceptible. The infective particles in the inoculum are unable to reproduce in such cells as a result of auto interference. If the proportion of insusceptible cells is high enough the rate of appearance of virus in the allantoic fluid of infected eggs becomes markedly reduced.[12]

With small inocula where the proportion of cells so blocked is very small the time required to double the virus concentration, i.e. the doubling time during the logarithmic phase in the allantoic fluid, is about 60 minutes.[12] But when the number of non infective particles in large inocula is sufficient to block 60% or more of the allantoic cells the doubling time becomes as long as the half life of infective particles. Then although the number of hemagglutinating particles increases in the allantoic fluid a large proportion of such particles appears to be noninfective.[12] To our way of thinking this finding does not provide evidence that the new particles represent immature or incomplete virus. As was stated earlier closely similar results are obtained when a sufficient number of heat inactivated particles is added to infective particles and the mixture is injected. Then also the rate of appearance and the yield of new particles is decreased and less than 10% of the particles in the yield are infective.

In contrast to what is obtained with large inocula containing a high proportion of non infective particles are the results secured with fully infective preparations. Our findings show that when large numbers of infective particles, i.e. more than 10^{8}, and only small numbers of non infective particles are injected more than 80% of the particles in the yield are infective at the time they are released from infected cells. Under these conditions auto interference does not occur and non infective particles appear only in small proportions.[12]

Such results underline the potential effects of interference in experiments on the mechanism of reproduction of influenza viruses. They indicate that non infective particles in sufficient number markedly alter the kinetics of multiplication and lead to exceptional results. In our opinion the simplest explanation for the high proportion of hemagglutinating but non infective particles obtained with the usual large inocula is spontaneous inactivation of infectivity. Such particles appear to represent a product of decay.

Regarding the second of the two phenomena under discussion, inhibition of virus reproduction by chemical compounds, there is now some fairly solid information. When host tissues previously infected with some viruses are brought in contact with certain chemical substances the yield of virus particles is diminished. As shown with bacterial viruses the addition of 5-methyl tryptophane[16] or proflavine[17] during the latent or eclipse period

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studies during single cycles of multiplication in surviving membranes *in vitro* show that the compound increases the duration of the latent period by about 80% reduces the yield of virus particles by about 99% but does not much alter the rate of appearance of new particles[30] Addition of the substance during the first third of the latent period causes inhibition of reproduction which is inverse in extent to the time of addition But as much as 80% inhibition is secured even when the compound is added during the last two thirds of the latent period[30] Inhibition is not obtained when the substance is added after completion of the latent period

In allantoic membrane cultures a concentration of 13×10^{-4} *M* of the 2 5-dimethyl compound causes 75% inhibition of influenza virus multiplication Certain other alkyl derivatives of benzimidazole e g the 2-ethyl-5-methyl compound are about ten times more active as inhibitors[30] The extent of inhibition appears to be related to the concentration of the compound used over a narrow range In the presence of the 2,5-dimethyl compound reproduction of the virus is held at a very low level for at least 70 hours[29] The demonstration of inhibition is not dependent on the measurement employed and the yield of both infective and hemagglutinating particles is markedly diminished[30 29] The effect of the compound on the membrane is not persistent and on removal of the substance membranes rapidly regain their capacity to support full reproduction of the virus Because the O consumption of the membrane is unaffected by the compound at inhibitory concentrations it appears that the effect is not dependent upon an alteration of oxidative metabolism[25]

As with *K pneumoniae* polysaccharide indications of the mode of action of 2 5-dimethylbenzimidazole have emerged from kinetic studies during single cycles of virus reproduction[0] Because the compound has no effect on influenza virus itself and does not affect either adsorption or release of the agent[3] it is assumed that the substance alters the reproductive process *per se* The structural relationship of the compound to adenine and to the benzimidazole moiety of vitamin B_{12}[28] has raised the possibility that it may act through an effect on nucleic acid metabolism Direct evidence bearing on this hypothesis has not yet been obtained Attempts to secure indirect evidence through experiments with likely metabolites have yielded no positive results and efforts to block the inhibitory activity with purines vitamin B_1 etc so far have been unsuccessful[30]

At the level of the susceptible cell it is obvious that neither *K pneumoniae* polysaccharide nor 2 5-dimethylbenzimidazole prevent infection and that the primary steps in the reaction between virus particle and host cell proceed as effectively in the presence as in the absence of the inhibitory substance Because either compound alters the reproductive process after infection of the cell has occurred it becomes evident that the outcome of a virus infection is not immune from external influence and may be modified in favor of the cell It has been feasible to test the potential therapeutic

reproduction of PVM [4] and mumps virus[25] when given during the latent period [19] [1] In single cycle experiments with large inocula the substance may be given 3 hours after mumps virus and 10 hours after PVM and inhibits multiplication In the latter case the interval is equal to about two thirds of the latent period [2] The yield of virus at the end of the cycle is diminished by 90% or more whether infective or hemagglutinating particles are used for measurement Only a single injection of a small amount of the substance is required and a few micrograms *i e* 2 to 5 causes inhibition with either of the viruses [4] In both instances the extent of inhibition is not proportional to the amount of polysaccharide used and after a single injection the effect of the compound as well as the substance itself persists in the tissue for a week or more [6]

As in virus interference there is evidence of specificity in chemical inhibition of multiplication Small quantities of *K pneumoniae* polysaccharide interrupt PVM and mumps virus reproduction in the mouse lung and allantoic sac respectively but large amounts do not affect the multiplication of influenza or Newcastle disease viruses in either tissue [4 2] There is a correlation between the results of chemical inhibition and virus interference experiments with the four agents mentioned [7 14] Thus the polysaccharides either interrupt the reproduction of both viruses in a pair showing interference or they fail to block that of either one Moreover with pairs of viruses which do not give interference the substance interrupts the reproduction of only one virus not that of the other [7] Although these findings provide no indication of the biochemical basis for inhibition of multiplication or interference they suggest that some facets of both are similar

The fact that the reproduction of some animal viruses can be interrupted by chemical means during the latent period bears on the mode of action of the inhibitory compounds The viruses themselves are unaffected by the polysaccharide infective particles are not inactivated and hemagglutinating particles are not inhibited [4 25] Obviously the adsorptive process can be excluded from consideration for it is completed before the compound is added Because the substance does not alter the rate of accumulation of virus when given near the end of the latent period an effect on the release process also can be excluded [19] In addition only small numbers of new virus particles are found in infected tissues treated with the compound no matter how the cells are ground or extracted As to the mode of action there remain for consideration only events occurring during the latter half of the latent period and it may be assumed that these are biosynthetic processes concerned with the reproductive process With *K pneumoniae* polysaccharide it has not yet been possible to penetrate more closely to the mechanism of inhibitory action

As demonstrated recently in our laboratory by Tamm *et al* [0 28 29 30] 2 5–dimethylbenzimidazole inhibits the reproduction of influenza viruses when the compound is added during the latent period The results of kinetic

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effect of *K pneumoniae* polysaccharide in mice infected with PVM the *smallest of the three animal viruses studied Although the substance is with*held for 3 days after infection and only 20 micrograms is given when evidence of pneumonia is already present the disease is modified and 60% or more of animals recover from an infection which is uniformly fatal in controls [7]

If it is assumed that the tissue damage resulting from a virus infection is due to abnormalities in cell function occasioned by reproduction of the agent then it is reasonable to think that compounds which inhibit the reproductive process might have therapeutic usefulness Similarly if it is thought that tissue damage is attributable to the cytotoxic effects of a high concentration of virus particles then compounds which decrease the yield of virus might also be useful It is clear that the results obtained with *K pneumoniae* polysaccharide in mice infected with PVM do not distinguish between these alternatives Emphasis should be placed upon the fact that once virus concentration has reached maximal levels in the lung the compound does not alter the progress of tissue damage or modify the course of the disease [4] Thus in this case the interval of usefulness is restricted and does not extend throughout the disease process

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29

Bacterial Transformation as an Infection by Desoxyribonucleic Acid

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The concept of viral infection includes two rather dissimilar parts the invasion aspect and the aspect of replication or proliferation The first mentioned offers so many different manifestations that virologists often seem to find little common ground when discussing different viruses in their different hosts with their varied modes of transmission vectors portals of entry and dormant phases What may add to the confusion is the unfortunate fact that the invasive aspect is also spoken of almost interchangeably as the infective process even though it is only a part of the whole process of infection the same word being understood to include the whole when used at other times There appears to be however a genuine common interest in the replicative mechanisms and less difference of opinions perhaps because there is a far smaller body of data about actual happenings in proliferative processes about which to disagree Bacterial transformation is another example of an invasive and replicative series of events and it is hoped that a brief treatment of this phenomenon may suggest some considerations useful in the virus field

Transformation as used in bacteriology signifies the permanent modification of one bacterial strain by cellular products derived from another strain By far the most important contribution in this field was made by Avery and coworkers who reported in 1944[1] that the transforming agent inducing a specific capsule type (Type III) in pneumococcus was closely associated with the high molecular desoxyribonucleic acid (DNA) of the encapsulated strain

In describing the typical bacterial transformation the following points are important

1 Transformation occurs when suitable bacteria are grown in the pres-

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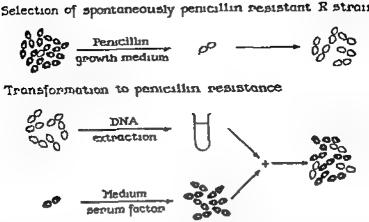

FIG 1

indicates in schematic fashion the process by which resistant cells resulting from spontaneous mutation can be selectively cultivated in the drug to which they are resistant and their parent strains are sensitive As indicated the DNA of such a selected strain gives rise to drug resistant transformants in a population which has never been exposed to the drug The progeny of these new cells inherit their resistance and continue to produce more DNA with the same potentiality

Here can be seen the aspects of DNA which make it reminiscent of an intracellular infectious agent As a biologically active entity it has been extracted from a cell which harbors it and after an indefinitely prolonged extracellular phase during which it does not multiply and is relatively stable it can again be introduced into suitable susceptible cells in which it will be propagated indefinitely producing modification of the host cell while it does so

Quantitative study of drug resistance transformations also throws some light upon the genetic aspects of the process Like genes the transforming factors for capsule synthesis and drug resistance act separately without any particular interference giving separate transformations even when they come from the same cells Thus the DNA from a strain of pneumococcus possessing both a specific capsule and one or another drug resistance will give rise to drug resistant transformants and to encapsulated ones but never in general to cells which have acquired both factors at the same time This result clearly suggests that it is the determinants rather than some components or cofactors of the enzymatic apparatus of the cells which are being transferred in the DNA

The specificity of the pattern is more striking still when the quantitative levels of drug resistance are considered Penicillin resistance is known to be

ence of specific transforming factor derived from a different donor strain of the same species

2 The transforming agent can be identified as DNA (since demonstrated more rigorously to be devoid of protein) [illegible]

3 The result of the transformation is to cause certain few cells of the recipient strain more nearly to resemble the donor strain

4 The progeny of the transformed cells continue to exhibit the induced properties indefinitely

5 The progeny of the transformed cells can in multiplying produce unlimited amounts of DNA capable of transforming other cells in the same way

The classic demonstration of these principles was soon followed by other specific capsule transformations in *Diplococcus pneumoniae*[4,5,6,7] and eventually in *Hemophilus influenzae*[8,9] and *Neisseria meningitidis*[10] also one obtained with partial regularity in a particular strain of *Escherichia coli*[11] since lost Transfers of certain drug resistance[3,1] [illegible] and a few enzymatic[14,15,16] properties have also been obtained these like all of the capsular and antigen transformations being induced by a high molecular DNA prepared in varying degrees of purity from strains of the homologous species bearing the specific property being transferred Altogether some forty different transformations involving about thirty biochemically different properties have been demonstrated in the several species

Of especial interest in connection with viruses are the reported transfer of virulence for plants between different strains and even species of crown gall bacteria[17] and conversion of rabbit fibroma into myxoma virus[18,19] although in these cases the nature of the change and of the extracts inducing it are not adequately understood A number of specific relatively well known bacterial characteristics have been shown to be transferred by viruses recovered from specific strains of *Salmonella paratyphi*[20,21] and *S typhosa* when they act upon bacteria of these same species Nothing more at present is known to suggest why an occasional virus particle succeeds in these transductions in carrying a host trait from cell to cell It is altogether possible that the virus sometimes transports a bit of host DNA or a DNA containing fragment of the host s genetic apparatus although there is no evidence for this view In any case the 10 000 fold greater frequency of transformations in pneumococcus (and 10 to 100 fold in *Hemophilus*) above that found in *Salmonella* brought about by DNA preparations that have given no indication of containing virus (or indeed protein) make it seem more reasonable to expect the *Salmonella* phage occasionally to bear host DNA than for the pneumococcus DNA to bear large quantities of an hypothetical exceptionally rugged protein free virus

Most useful in investigation of the nature of these processes have been the drug resistance transformations which allow the newly transformed (resistant) cells to be selected out and quantitatively determined[12] Figure 1

At the same time each of these single DNA factors can be looked upon as an independent agent capable of invading producing a modified cell and being reproduced indefinitely like one of the better adapted infectious agents This aspect is brought out in experiments upon the rate of develop ment of drug resistant transformants Figure 3 shows a typical curve This reveals that after a very short exposure to DNA carrying drug resistance factor there is a brief latent phase during which no resistance is detectable Then within very few minutes many thousands of transformants abruptly reveal themselves with a fully established drug resistance These cells do not however multiply even in the absence of the drug for a considerable time and then at last begin to do so

Rate of drug resistance transformation

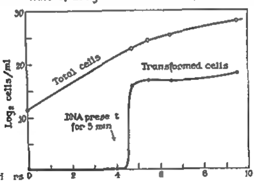

FIG 3

Considering the process as mimicking an infection the first horizontal phase is the one during which the cells become susceptible to DNA Then there is a distinct latent period before the cell demonstrates the modification which is initiated by the short exposure to DNA After this modification (drug resistance) has been completed there is a further latent phase during which the modified cell learns to reproduce itself and at the same time the new DNA as well

Considered again from the other point of view the results also seem to have genetic significance The early period during which drug resistant cells are appearing seems to represent the time when the gene is initiating its phenotypic expression The plateau and later rise represent the period when the gene is coming to be reproduced itself Thus the two fundamental properties of a gene appear to be displayed and separated in time from each other *in the later stages* of transformation

One may perhaps reconcile the infectious and the genetic views of transformation If DNA proteins (and occasionally ribosenucleoproteins) constitute the genetic apparatus of most species then it is these entities most of all which have to be duplicated and passed on if there is to be

acquired in stepwise spontaneous mutations in bacteria each step permitting growth in a higher concentration of the drug (pattern A Figure 2) [23] When a highly resistant pneumococcal strain which has undergone multiple successive steps of mutation to resistance is the source of transforming agent penicillin sensitive cells are transformed only to the first lowest level of resistance [11] The product of this one step process treated with the same DNA from the multiple resistant strain can now acquire the second step of resistance and so on progressing upward along curve A In other words transformation has transferred in stepwise fashion genetic determinants which correspond exactly to a series of determinants which had previously

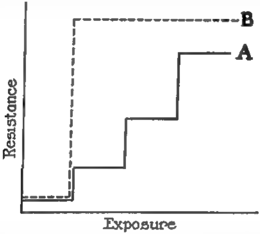

FIG 2

been successively acquired in the spontaneous mutations undergone by the donor strain Streptomycin resistance also appears in quantitative mutational steps transferred stepwise in transformation Here however besides the stepwise resistance series (type A) there is known a more rare mutation which gives in a single step a highly streptomycin resistant mutant (pattern B Figure 2) If we take two strains one highly resistant as the result of multiple cumulative mutations the other as the result of the single high step we can prepare two DNA transforming agents from two strains of different genetic makeup but outwardly similar properties It is observed that each DNA preparation unmistakeably reflects the history of its donor strain by transmitting the first level of resistance that strain had reached which in one case (strain type A) is a low step in the other (type B) a high step Again we are impressed with the fact that the DNA represents not merely the attained resistance state of the donor bacteria but actually appears to be a repository for the successive genetic experiences of the donor strain It can be concluded that the DNA is not only the transmissible form of the bacterial mutations but also the site in which they had occurred and therefore the very material of which the genetic apparatus is made

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reproduction of the species When a bacterial or multicellular species does this it is called growth and reproduction When an intracellular virus accomplishes the same thing it is more likely to be called infection Although in their more complex manifestations genetic and infectious phenomena can be rather clearly distinguished at their most elementary levels these two principles appear to find common expression in the processes in which nucleic acids take part Thus as we have already seen there are both genetic and infectious aspects to the events that follow the introduction of coliphage DNA into host bacteria The infection in fact seems to be achieved by the commandeering of the genetic apparatus of the host cell Doubtless DNA entities better adapted to the host cell underlie the less drastic infections with the symbiotic viruses At a perhaps still simpler level the single factor transforming DNA s of bacteria have the same dual aspect These agents invade a suitable cell with such precise adaptation and with so small a change in the overall metabolism that we are accustomed to think of the infected strain as perhaps genetically unique but well within the range of what is normal biologically Since this seems reasonably justified transformation then appears as the elemental unit process of normal genetics—a single factor transfer of a small well adapted homologous unit As the homologous unit becomes larger and more complex we approach more and more the domain of classical genetics as the unit becomes more and more abnormal or foreign we come closer to the border line of pathology and infection

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ing at least limited success in a search for inhibitors active against poliomyelitis virus

Stimulated by the observations (1) that tryptophan deficiency resulted in a particularly marked prolongation of incubation periods in mice infected with Type II poliomyelitis or Theiler s GDVII encephalomyelitis [17 9] and (2) by the earlier discovery that 5-methyltryptophan inhibited the growth of coliphage [3] we undertook a study of a number of substituted methyl tryptophans[18] and other tryptophan antagonists* for prophylactic activity against Type II poliomyelitis in mice [0] Mice receiving adequate amounts of protein tolerate high levels of these tryptophan derivatives well and one of the compounds 6-methyltryptophan Figure 1 can be fed to mice in a tryptophan deficient diet without greatly aggravating the deficiency Our earlier study of these analogs was limited by the amounts available to an inconclusive experiment with small numbers of animals and Theiler s GDVII virus The results suggested however that incubation periods in mice receiving 6-methyltryptophan were prolonged to a greater extent than in mice with tryptophan deficiency alone

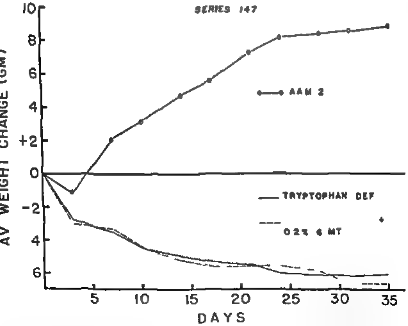

FIG 1 Growth curves of mice on complete AAM-2 tryptophan deficient and tryptophan deficient plus 6-methyltryptophan diets

We w h to cknowledge the generous a si tance of Dr H ■ Snyder of the Department of Chem try The Univers ty of Illinois and of Charles Pfizer and Company fo the synthesis of tryptophan derivatives

30

Nutritional Inhibitors

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The possibility that antiviral agents might be discovered among nutritional inhibitors is naturally suggested by the observation that mice suffering from nutritional deficiencies are less susceptible to certain viral infections than are well nourished controls [6] The administration of oxythiamine to mice inoculated with Lansing poliomyelitis[13] resulted in a significant delay in the onset of paralytic disease but the end result was essentially that previously observed in thiamine deficiency i e all treated mice ultimately succumbed Similarly desoxypyridoxine in small doses during the incubation period exerted some protective activity in infections with the pneumonia virus of mice PVM [14,1] but the administration of desoxypyridoxine for a period prior to infection actually appeared to enhance the severity of *infections with PVM virus The methionine antagonists* ethionine and methoximine inhibited the growth of influenza[1] in tissue culture another methionine antagonist methionine sulfoximine also inhibited influenza virus and poliomyelitis virus in tissue culture[4] and in mice [3,10] Yet a prophylactic or therapeutic activity has not been demonstrated Morgan[16] has described antiviral activity of several folic acid antagonists against psittacosis virus in tissue culture but reports of similar experiments in intact *animals have not appeared We have ourselves*[7] *tested a variety of folic* acid antagonists against Type II poliomyelitis in mice without success

When the potentially adverse influences of nutritional stress on resistance to infection are considered particularly development of maturation resistance and the impairment in antibody production [1] one may question whether or not continued interest in this approach to the chemotherapy of viral infections is warranted In spite of these theoretical objections we have thought that nutritional inhibitors might be discovered which would interfere selectively with the growth of viruses I shall present data suggest

of Lansing poliomyelitis in such mice is essentially similar to that in well nourished mice with most of the infections developing within two weeks The addition of 0 2% 6–methyltryptophan to this diet a 4 to 1 ratio of analog to tryptophan results not only in an increase in incubation period greater than that seen in tryptophan deficiency alone but also in the survival of a significant number of mice The addition of 6–methyltryptophan in a ratio of 10 to 1 0 5% has an even more pronounced effect with half of the mice surviving Furthermore most of the survivors remain well after being placed on a high protein (19% casein) diet without 6–methyltryptophan and resist intracerebral challenge with 500 LD_{50} of Lansing virus given one month after the withdrawal of 6–methyltryptophan This shows that an active immunity develops even though symptoms of poliomyelitis are suppressed

The question may well be raised however Is the suppressive action simply that of a partial tryptophan deficiency induced by the antagonist? If so then a similar sparing action should be demonstrable in mice at some level of tryptophan deficiency Table 1 summarizes the results of an experiment[23] in which groups of mice were fed decreasing levels of tryptophan and it is seen that at no level is there a protective influence approaching that observed in the group receiving 6–methyltryptophan

Table I

COMPARISON OF INFLUENCE OF GRADED TRYTOPHAN DEFICIENCY AND OF 6–METHYL TRYPTOPHAN ON LANSING POLIOMYELITIS IN MICE

Diet	*No Mice*	*Deaths in Nutritional Controls*	*Average Incubation Period* (Days)	*Average Survival Time* (Days)
Controls Complete Diet	1	0/7	6 6	7 5
Tryptophan Deficient	20	3 7	8 7	10 4
0 01% Tryptophan	20	1/6	[illegible]	17 1
0 03% Tryptophan	20	0/7	9 6	11 2
0 05% Tryptophan	19	0 7	8 4	9.9
0 05% Tryptophan plus 0 5% 6–Methyltryptophan	20	0 7	15 5	17 8

Mice inoculated after seven days on experimental diets with 100 LD_{50} Type [illegible] (Lansing) poliomyelitis virus intracerebrally

I shall now describe briefly preliminary experiments[20] indicating that the ingestion of low tryptophan diets containing 6–methyltryptophan may also result in some protection against oral infection with Type I poliomyelitis virus in cynomolgus monkeys *Our studies in mice had shown that the* greatest protection against Lansing infections was observed in the animals fed diets containing a small amount of tryptophan (0 05%) and ten times that amount of 6–methyltryptophan The tolerance of 1 2 to 2 2 kg Philippine cynomolgus monkeys for 6–methyltryptophan was approximated by

We have since been able to show[19] that in Lansing infections in mice 6–methyltryptophan has a suppressive effect which appears to be more selective than other inhibitors employed in our laboratory On a control diet, consisting of a mixture of amino acids AAM–2 (Figure 2) in place of protein almost all animals develop typical paralytic disease and die during the first 14 days after intracerebral inoculation with 100 LD_{50} of Lansing virus On tryptophan deficient diets an increase in the incubation periods occurs with the peak incidence of paralysis and death between the tenth and twentieth days The addition of 0 2% of 6–methyltryptophan to a deficient diet results in a much greater delay in the onset of the experimental infection the peak falling in the fourth week In a typical experiment the paralysis rate did not exceed 60% at the end of the observation period there was however a compensatory increase in deaths in mice in which paralysis was not observed possibly due to acute tryptophan deficiency precipitated by the infection the analog or both In any case although the incubation period was greatly prolonged the final result was not influenced favorably

If the 6–methyltryptophan is added to a ration containing a small amount 0 05% of tryptophan the result is quite different Mice receiving this amount of tryptophan do not grow rapidly but they remain alert and active and do not show evidence of acute tryptophan deficiency The pattern

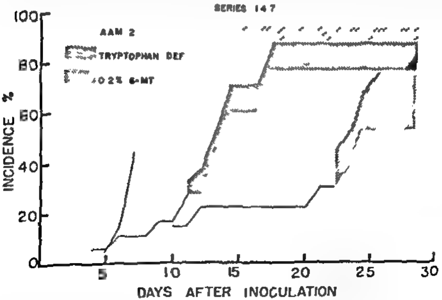

Fig 2 Lansing poliomyelitis in mice on complete AAM–2 tryptophan deficient and tryptophan deficient plus 6–methyltryptophan diets The upper contour of each curve indicates cumulative deaths the lower contour cumulative paralysis

Philippine cynomolgus monkeys weighing from 1 2 to 2 2 kg and the Wisconsin 45 strain of Type I poliomyelitis virus in the form of a pool of 10% monkey brain-cord suspension prepared and stored in 50 ml aliquots at —40 C were employed The monkeys were inoculated by offering them 20 ml of virus suspension sweetened with sucrose in their drinking cups Water was withheld on the day or days virus was given until the virus suspension was consumed Ordinarily the animals drank the virus suspension immediately The indicated amounts of 6–methyltryptophan and tryptophan were added to the dry corn grit basal moistened with water and cooked in the autoclave for 30 minutes at two pounds pressure The resulting ration was granular but soft and it was much more palatable to the monkeys than a dry ration The ration was offered in small amounts about 50 grams three times daily Control animals received the 18% casein basal

In the first experiment a group of nine monkeys was placed on the basal ration containing 2% 6–methyltryptophan and 0 05% DL–tryptophan for seven days on the eighth ninth and tenth days this group and a control group of nine monkeys were fed a total of 120 ml of 10% virus suspension (about 1 200 000 intracerebral LD_{50}) in six doses of 20 ml each Temperatures were recorded daily and the animals were observed for signs of disease twice daily Three weeks after the first virus feeding the surviving animals were placed on the 18% casein basal diet The results are recorded in Table 3

Table 3

ORAL INFECTION WITH TYPE I POLIOMYELITIS VIRUS IN CYNOMOLGUS MONKEYS RECEIVING 6–METHYLTRYPTOPHAN (6–MT)

2% 6–MT 0 05% Tryptophan in Corn Grit Basal			*M 3 Control*		
Monkey No	*Fever†*	*Paralysis‡*	*Monkey No*	*Fever†*	*Paralysis‡*
1	0	8	17	5 6	6
6	0	8	24	4 5 6 7	7
11	0	8	16	5 6 7	8
8	7 8	9	21	7	8
15	9	9	20	8	8
9	9	10	18	7,8	9
13	0	10	2	8 9 1?	9
3	0	0§	23	8	9
10	0	0§	[illegible]	8	11
TOTAL PARALYSIS		7			9

Monkeys were fed experimental rations for seven days on the eighth ninth and tenth days each animal received two 20 ml feedings of 10% cord suspension of Wisconsin 45 virus (a total of 120 ml containing 1 200 000 intracerebral LD)

† Fever—Days after first virus feeding on which fever of 103 F or higher developed

‡ Paralysis—Days after first virus feeding when paralysis appeared

§ Monkeys No 3 and No 10 resisted intracerebral challenge with 500 LD Wisconsin 45 virus six weeks after oral inoculation

feeding 6–methyltryptophan in a diet consisting of corn grits 89% corn oil (fortified with oleum percomorphum 25 ml per liter) 5 5% salts IV of Phillips and Hart 4% 1–lysine HCL 1 0% and DL–methionine 0 5% Water soluble vitamins were provided as a supplement in the drinking water each morning Control animals were fed our basal ration (M–3) containing 18% casein The addition of 2% 6–methyltryptophan to the corn grit basal either with 0 05–0 1% supplementary tryptophan or without had no consistent adverse influence Table 2 Some animals lost weight rapidly on these rations but so also did animals on the corn grit basal without 6–methyltryptophan occasional animals failed to thrive even on 18% casein

Table 2

CHANGES IN WEIGHTS OF CYNOMOLGUS MONKEYS FED 2% 6–METHYLTRYPTOPHAN IN CORN GRIT BASAL FOR FOUR WEEKS

Experiment 1			*Experiment 2*			*Experiment 3*		
No	2% 6–MT 0 05% tryptophan		No	2% 6–MT no added tryptophan		No	2% 6–MT no added tryptophan	
	O W *	*F W* †		*O W*	*F W* †		*O W* *	*F W* †
3	2292	2203	25	1337	1216	49	1612	1575
10	1456	1583	26	1205	1110	50	1122	11[illegible]0
			27	1375	1010	51	1304	1244
			28	1372	1250	52	1198	1008
			29	1245	1165	53	1393	1541
			30	1460	1260	54	1441	1450
			32	1252	1030	55	1670	1700
			33	1550	1415	56	1841	1870
			34	1770	1555	57	1299	1255
						58	1340	13[illegible]6
			Avg	1398	1223		1427	1399

* Weight in grams when placed on ration indicated

† Weight in grams four weeks later

Subsequent nutritional observations were limited to those animals which also received virus and definite conclusions as to the chronic toxicity of 6–methyltryptophan cannot be drawn It would appear that as has been shown to be true in the mouse 6–methyltryptophan is not a highly active nutritional antagonist for tryptophan in the monkey Several of the monkeys developed anorexia during the last days of the experiment and it is thought that this may have been a manifestation of chronic tryptophan deficiency On the other hand it is probable that some animals would tolerate 2% 6–methyltryptophan in the corn grit basal for a longer time and that concentrations considerably greater than 2% could be fed for shorter periods Although a general tendency to diarrhea was observed among all of the monkeys including those receiving 18% casein none developed the severe dysentery characteristic of deficiencies of folic acid and other water soluble vitamins

Table 5

ORAL INFECTION WITH TYPE I POLIOMYELITIS VIRUS IN CYNOMOLGUS MONKEYS RECEIVING 6–METHYLTRYPTOPHAN (6–MT)

2% 6–MT in Corn Grit Basal			*M–3 Control*		
Monkey No	*Fever†*	*Paralysis**	*Monkey No*	*Fever†*	*Paralysis**
57§	7	0	67	3 6 10	10
51	6 7,8	0	62	9 10 11	12
53	6 7 13	0	64	12	13
49	0	0	63	3 1*	30
50	0	0	59	3	0
5	0	0	68	4	0
54	0	0	66	0	0
55	0	0	61	0	0
56	0	0	65	0	0
58	0	0			
TOTAL PARALYSIS		0			4

Monkeys were fed experimental diets for seven days on the eighth day each animal received two 20 ml feedings of a 1 40 dilution of stock 10% cord suspension of Wisconsin 45 virus (a total of 40 ml of a 1 400 dilution of infected tissue containing 10 000 intracerebral LD)

† Fever—Days after first virus feeding on which fever of 103 F or higher developed

* Paralysis—Days after first virus feeding when paralysis appeared

§ Monkey No 57 was slow dull and ataxic nine days after inoculation with virus but recovered without developing paralysis

6–methyltryptophan is highly insoluble Effective assimilation from the alimentary tract might be expected to require the simultaneous presence of other amino acids[5 11] and in fact Gebhardt and Bachtold [10] find that large doses of 6–methyltryptophan fail to protect monkeys which have been starved for 48 hours prior to inoculation The fact remains however that susceptibility to paralytic poliomyelitis in the monkey as well as in the mouse can be modified by the administration of an amino acid antagonist

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Experiments 2 and 3 were similar except that (1) the 2% 6–methyl tryptophan ration was not supplemented with tryptophan and (2) the monkeys received two feedings of 20 ml of a 1 400 dilution (approximately 10 000 intracerebral LD_{50}) from the same pool of Wisconsin 45 virus on the eighth day of the experiment The surviving animals of all three experiments were challenged intracerebrally with 500 LD_{50} Wisconsin 45 virus 6–9 weeks after their oral exposure

In Experiment 1 Table 3 there was a suggestion of protection against the virus in the 6–methyl tryptophan group i e incubation periods were longer febrile prodromata less pronounced and two animals escaped paralytic infection These two animals Nos 3 and 10 apparently did experience an inapparent infection for they resisted a large intracerebral challenge [illegible] weeks later In the second Table 4 and third Table 5 experiments the smaller inoculum resulted in a lower incidence of paralytic infection in the controls 8/10 and 4/9 respectively while only one of the twenty animals receiving 6–methyltryptophan became paralyzed The surviving animals in the latter experiment succumbed to intracerebral challenge

These observations must be interpreted with caution There are many factors which would tend to limit or even preclude practical application

Table 4

ORAL INFECTION WITH TYPE I POLIOMYELITIS VIRUS IN CYNOMOLGUS MONKEYS RECEIVING 6–METHYLTRYPTOPHAN (6–MT)*

2% 6–MT in Corn Grit Basal			*M–3 Control*		
Monkey No	*Fever†*	*Paralysis‡*	*Monkey No*	*Fever†*	*Paralysis‡*
31	5	7	37	6	7§
32	3	0	43	6 7	8
25	0	0	36	5 7 8	8
26	0	0	41	9	10
27	0	0	38	6 8 9 10	11
28	0	0	42	7	12
29	0	0	35	0	14
30	0	0	44	0	19
33	0	0	39	0	0
34	0	0	40	0	0
TOTAL PARALYSIS		1			8

* Monkeys were fed experimental diets for seven days on the eighth day each animal received two 20 ml feedings of a 1 40 dilution of stock 10% cord suspension of Wisconsin 45 virus (a total of 40 ml of a 1 400 dilution of infected tissue containing 10 000 intracerebral LD_{50})

† Fever—Days after first virus feeding on which fever of 103 F or higher developed

‡ Paralysis—Days after first virus feeding when paralysis appeared

§ Monkey No 37 was found dead on the seventh day Examination of the brain and cord revealed typical histopathology of severe poliomyelitis

31

Inhibition of Virus Multiplication through Con sidered Use of Antimetabolites

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The purpose of this paper is to demonstrate how it is possible to predict and to realize antiviral agents which will harm the invading organism without ■ the same time poisoning the animal host The selective action which such compounds exhibit is a prime requisite of a useful chemotherapeutic drug Without it no chemical substance can be considered useful and practical in the treatment of disease because if the proposed drug is harmful to the animal host as well as to the invading microorganism treatment with the drug may well be as disastrous to the animal as is the untreated disease I wish to emphasize this point because it is an important one which has received practically no attention At most investigators of chemotherapy have been aware of the desirability of selective action but with a gesture almost of despair have resigned themselves to the task of finding antiviral agents in the hope that if they do find one it will perchance exhibit the desired selective toxicity In the earlier work on bacterial chemotherapy sulfanilamide and penicillin were found in this way as were also streptomycin and chloromycetin Balanced against these successes however are a surprising number of failures A great deal of effort in chemotherapy has terminated finally in failure because the antiviral or antibacterial agents found have proven to be toxic to the animals one wishes to treat

This afternoon I want to show you that it is not essential to rely blindly on chance to yield us antiviral agents of little or no harmfulness to animals It has been possible to make several laboratory test systems in which the chemical structure of a substance was predicted which would harm a given virus and would not harm the host These compounds have been synthesized and shown to function in the desired selective way I want to describe to you two separate cases in order to illustrate that there is not just one way

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31

Inhibition of Virus Multiplication through Considered Use of Antimetabolites

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The purpose of this paper is to demonstrate how it is possible to predict and to realize antiviral agents which will harm the invading organism without at the same time poisoning the animal host The selective action which such compounds exhibit is a prime requisite of a useful chemotherapeutic drug Without it no chemical substance can be considered useful and practical in the treatment of disease because if the proposed drug is harmful to the animal host as well as to the invading microorganism treatment with the drug may well be as disastrous to the animal as is the untreated disease I wish to emphasize this point because it is an important one which has received practically no attention At most investigators of chemotherapy have been aware of the desirability of selective action but with a gesture almost of despair have resigned themselves to the task of finding antiviral agents in the hope that if they do find one it will perchance exhibit the desired selective toxicity In the earlier work on bacterial chemotherapy sulfanilamide and penicillin were found in this way as were also streptomycin and chloromycetin Balanced against these successes however are a surprising number of failures A great deal of effort in chemotherapy has terminated finally in failure because the antiviral or antibacterial agents found have proven to be toxic to the animals one wishes to treat

This afternoon I want to show you that it is not essential to rely blindly on chance to yield us antiviral agents of little or no harmfulness to animals It has been possible to make several laboratory test systems in which the chemical structure of a substance was predicted which would harm a given virus and would not harm the host These compounds have been synthesized and shown to function in the desired selective way I want to describe to you two separate cases in order to illustrate that there is not just one way

to realize selectively toxic substances One of these will deal with influenza virus and the other with spontaneous mammary tumors of mice

These examples are going to revolve around antimetabolites However, in the very beginning it should be emphasized that just any antimetabolite will not do A large amount of testing of miscellaneous antimetabolites for chemotherapeutic activity should have convinced everyone (although I am afraid it has not) that antimetabolites *per se* are not the magic words of chemotherapy Rather I think the promise of success lies in the understanding of antimetabolite action and the considered use of this knowledge

First let us discuss what constitutes an antimetabolite Certain chemical compounds have a structural resemblance to specific essential metabolites of living things and in addition these analogous compounds are able to counteract the vital processes which are carried on by these essential metabolites Such structural analogs which antagonize the action of essential metabolites are called antimetabolites The structural resemblance of metabolite and antimetabolite is important because it is this feature of the antagonist or inhibitor which allows it to exclude the essential metabolite from its vital functioning It is this exclusion which produces the biological effect The action of antimetabolites can be described simply as shown in the first slide The enzymic reactions which are the basis of most living processes take place in two steps as shown on the slide The antimetabolite works because while it can take the place of the metabolite in the first step it cannot in the second and like the fabled dog in the manger it prevents the normal metabolite from doing so too

A well known antimetabolite is sulfanilamide It has been demonstrated to exclude p–aminobenzoic acid from its normal enzymic course which is the synthesis of folic acid [1,2] As a result the organism dies from lack of folic acid This view of the matter has been boiled down to its essentials for the sake of clarity There is a variety of ancillary knowledge about it which however need not concern us here Nevertheless we must recognize that the scheme shown in Figure 1 is not an hypothesis because there is a body of experimental facts to substantiate the conclusion that sulfanilamide does inhibit the synthesis of folic acid in the manner shown

Let us now apply this understanding of antimetabolite action to the problem of finding an agent to inhibit the multiplication of influenza virus This was in fact done early in 1947 [3] but for reasons which will appear shortly I shall presume to recapitulate We need to know a specific enzyme of virus which it uses in its vital processes and we also need to know the specific metabolite which is the substrate of this enzyme Fortunately the concept of Dr Hirst[4,5] on the hemagglutination phenomenon provided both The enzyme is the hemagglutinin of the influenza virus and the metabolite or substrate is the virus receptor in the red cell and presumably also in susceptible lung cells We need to know the nature of the substrate but this is not entirely clear even today However in the winter of 1946–7

Hirst s evidence obtained with periodate oxidation suggested that it was a polysaccharide The idea thus arose in our laboratory that a suitable poly saccharide would prevent the interaction of virus and red cell which we see as hemagglutination The inhibitory polysaccharide if it were to function as an antimetabolite of the virus receptor would need to be related struc

Substrate + enzyme ↔ complex
Complex ↔ products + enzyme
Antimetabolite + enzyme ↔ inactive complex
Inactive complex ↔ no products
PAB + unknown substrate + enzyme ↔ complex
Complex ↔ folic acid + enzyme
Sulfanilamide + enzyme ↔ inactive complex
Inactive complex ↔ no folic acid

FIG 1

turally to but most certainly not be identical with this receptor From the knowledge that the polysaccharides of lungs are usually glucuronic acid conjugates it was a good guess that galacturonic acid-containing polysac charides many of which occur naturally in plants would inhibit the hemag glutination caused by influenza virus Apple pectin is such a galacturonic

Table 1

EFFECT OF APPLE PECTIN ON AGGLUTINA TION OF CHICKEN RBC IN PRESENCE AND ABSENCE OF INFLUENZA A VIRUS

Apple Pectin	*Hemagglutination*	
gamma per cc	*with virus*	*without virus*
6666	partial	partial
3333	partial	none
1666	partial	none
833	trace	none
208	trace	none
104	partial	none
26	partial	none
13	complete	none

acid-conjugate and trials in the laboratory soon showed that it did inhibit the hemagglutination of chicken erythrocytes by PR8 influenza virus This fact is demonstrated by the data in Table 1 Several other galacturonic acid containing polysaccharides exhibited the same property to a greater or lesser degree as can be seen from the data in Table 2 In this table it can also be seen that several other types of polysaccharides were inactive

Table 2

EFFECT OF VARIOUS CARBOHYDRATE CONTAINING MATERIALS ON HEMAGGLUTINATION BY INFLUENZA A VIRUS

Substance	*Least Inhibitory Concentration*
	γ per cc
Polysaccharides	
Apple pectin	26
Citrus pectin	104
Flaxseed mucilage	Less than 13
Gum acacia	Less than 13
Specific polysaccharide of acacia	Inactive at 6666
Alginic acid	833
Soluble starch	*Inactive at 6666*
Starch polyaldehyde	Inactive at 6666
Starch polyacid"	Inactive at 6666
Agar	Inactive at 52
Simple Carboyhdrates	
Galacturonic acid	Inactive at 6666
Cellobiuronic acid	Inactive at 6666
Inositol galactoside tartarate	Inactive at 6666
Galactose	Inactive at 6666
Aldobionic acid of flax	Inactive at 6666
Glucose	Inactive at 6666
Mannose	*Inactive at 6666*
Ribose	Inactive at 6666
Complex Concentrates	
Blood group A substance	Less than 13
Chicken RBC extract	Less than 13

These findings of the antiviral effect of apple pectin may now be cast in the scheme shown in Figure 2 The virus receptor is the metabolite or the substrate and the enzyme is the hemagglutinin of the virus Pectin is the

Substrate + enzyme ⇄ complex
Complex ⇄ products + enzyme
Antimetabolite + enzyme ⇄ inactive complex
Inactive complex ⇄ no products
RBC Receptor + viral enzyme ⇄ complex
Complex ⇄ products + enzyme
Apple pectin + viral enzyme ⇄ inactive complex
Inactive complex ⇄ no products

FIG 2

antimetabolite which bears structural analogy to the metabolite It is clear that the pectin acts by preventing the attack of the virus on the receptor Actual experiments failed to reveal viricidal properties for the pectin

This concept provides us with a sensitive and specific test for the receptor

If we were to inhibit hemagglutination with a fixed amount of pectin and were then to increase the concentration of the receptor, the action of the pectin in inhibiting hemagglutination should be overcome. This is the same sort of assay one uses for p-aminobenzoic acid. In that case one uses a fixed amount of sulfanilamide and measures the concentration of PAB by determination of how much of it will allow bacterial growth in the presence of the antimetabolite.

This has been done and it has been shown that a substance can be extracted from red cells of human beings and that this substance will antagonize the action of apple pectin.[8] Furthermore, the antagonism was competitive in nature, just as it should be if the pectin is an antimetabolite of the receptor contained in the extracted material. A correlation was found to exist between the susceptibility of an animal species to influenza infection and the quantity of this pectin antagonist which could be extracted from erythrocytes. If a given species was susceptible, the antipectin activity of the erythrocyte extract was large, and vice versa. Finally, the receptor substance or virus substrate was purified to a very large extent, and the purified substance was shown, by physical measurements, to react with highly purified influenza virus. The activity of this substance was very high, because in the presence of 0.75 mg. of pectin one could measure the effect on hemagglutination of 0.00004 gamma of it. If we bear in mind that this test is made with unheated virus, and not with a socalled indicator strain of non-infectious particles, the high potency is even more remarkable, and would seem far to exceed the activity of materials purified from salivary glands and other tissues by other workers, even when one compares the activities of these extracts by the use of indicator strains with the potency of the erythrocyte substance tested with live virus.

Not only will apple pectin inhibit hemagglutination by influenza A virus, but in addition it will prevent the multiplication of the virus in the allantoic chambers of embryonated eggs. The data of Table 3 will illustrate this fact. These data will also show that a polysaccharide such as alginic acid, which

Table 3

EFFECT OF APPLE PECTIN AND OF ALGINIC ACID ON MULTIPLICATION OF INFLUENZA A VIRUS IN EMBRYONATED EGGS

Substance	*Amount*	*When Given*	*Eggs*	*Eggs Showing Virus Multiplication*
None			24	24
Apple Pectin	50	before virus	61	5
		after virus	56	17
	25	before virus	4	1
Alginic Acid	50	before virus	16	14
		after virus	28	4

was ineffective in inhibition of hemagglutination was likewise incapable of retarding multiplication of the virus in the eggs

This chemotherapeutic action of apple pectin is noteworthy for several reasons One of these is that this property sets it apart from the various inhibitors of hemagglutination which have been extracted from animal tissues These latter which have been concentrated from eggs and from lungs from urine and from saliva, will not prevent multiplication of the virus The reason I think is clear as to why this difference should exist Let us refer again to Figure 2 It is widely believed that the inhibitors of viral hemagglutination isolated from animal tissues are either the virus receptor or a degradation product of it In any event these inhibitors are attacked and destroyed by the virus as many experiments have shown In the terminology of Figure 2 they are the virus substrate or a derivative of it It is now clear why they do not inhibit multiplication of the virus To expect them to do so is like expecting to stop an invading army by overfeeding it On the other hand one would expect an antimetabolite such as apple pectin to prevent multiplication of the virus It acts by excluding the virus from its substrate and the reaction of the virus with its substrate is essential to the multiplication and other vital processes of it One can readily demonstrate that the virus does not destroy apple pectin

It is now important to say that apple pectin has a selective toxicity It prevents the action and the multiplication of the virus and it does so without being toxic to the embryonated eggs We may well inquire why this is so We have seen why it is harmful to the virus It is relatively clear also why it is harmless to the host There is a body of evidence which time will not permit us to discuss now which indicates that the pectin prevents the invasion of susceptible cells by the virus In other words it prevents the entrance of the virus into the cell The polysaccharide like virus receptor is believed to exist at or near the surface of cells where it probably serves a structural or anatomical function In other words it is part of the cell wall The virus must penetrate this wall to invade the cell and to multiply It probably passes the wall by enzymic hydrolysis of it brought about by its special enzyme which we call its hemagglutinin This enzymic reaction is susceptible to inhibition by apple pectin in the manner we have pictured On the other hand the polysaccharide like receptor is an anatomical unit of the cell not designed for active metabolic (that is to say enzymic) reactions Since there is no need for the cell to metabolize the receptor it can tolerate any amount of an antimetabolite of it

Thus we can see quite clearly why apple pectin has a selective and antiviral effect The selective toxicity is achieved by taking advantage of the different roles played by the virus receptor with regard to the virus and to the animal cell

We have come this long way and have used up most of the allotted time in order to see clearly the principles involved in this rather simple demon

stration I think it has been worth while if it has served to illustrate the thesis of this paper namely that it is entirely possible to achieve selectively toxic compounds if we but put to use existing knowledge It is by no means a hopeless task Many points in the work concerning the actual manipulations have been slighted from lack of time Some of these points are absolutely essential to the demonstration I would therefore urge you all to read the published accounts of the work in order to gain a fuller understanding I would also like to dispel a rumor which has just recently been published in one of the journals to the effect that apple pectin does not work as has just been described In fact the statement was made that it does not work at all [7] I want to say that in our experience there is no difficulty at all in reproducing the results which I have been describing to you and that we will always be glad to demonstrate the inhibition of hemagglutination caused by apple pectin to any one who doubts it.

As was stated earlier the work just described was published in 1947 and 1949 Despite this age of the data I have brought them to your attention today because I feel that there is much in them which has escaped the notice of virologists and which is of considerable importance in attempts at chemotherapy of the disease Apple pectin itself is not active enough on a weight basis to be considered for practical use in medicine but the principles illustrated by the experiments may well be of much importance

In the few minutes which remain I would like to discuss with you a second case in which an antiviral agent has been predicted and realized This one is of much more recent vintage but like the first it involves an agent which is essentially harmless to the animal host while being harmful to the invader This is the case of the temporary obliteration of spontaneous mammary cancers of mice with antimetabolites of dimethyldiaminobenzene I think this case is important because it shows a second way in which selectively toxic agents can be realized There are probably many other ways yet to be found The only justification for bringing this case to the attention of virologists is that several investigators now believe as a result of Bittner's work that these spontaneous cancers of mice are caused by infection of the suckling young with a virus like living particle

Time will not permit a full description of the working out of this example but if any of you are interested the full story can be read in 3 papers published during the course of the past 3 years [8 9 10]

One of the crucial points on which this case depended was the finding of a metabolic difference between normal animals and spontaneous cancers I do not mean to say that glucose or amino acids were metabolized differently in the two for I fear that is the usual connotation of the words metabolic difference Rather the difference was that whereas normal animals depended on a dietary source of vitamin B_1 and apparently did not synthesize it themselves the spontaneous mammary cancers synthesized this essential substance There is thus a fundamental difference in the metabolic

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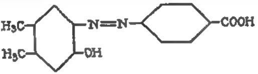
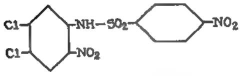
product reversal If a drug such as sulfanilamide inhibits an essential en zymic reaction and thereby deprives the cell of the product of the reaction then the supplying of the product should erase the drug-effect To avoid this phenomenon is not easy In fact until recently it has been impossible to do so except by chance It is known that sulfanilamide avoids it and that the chemotherapeutic potency of sulfanilamide would not exist without it A study of how sulfanilamide avoids it has now led to a way which allows us to make antimetabolites which likewise avoid it However the important point for us now is the one involving selective toxicity The selective action depended on the finding and taking advantage of the difference in metabolic machinery of host and parasite



CPA



DCDNS

Fig 5

The antimetabolites are those shown in Figures 4 and 5 It should be emphasized that they are not antivitamins–B_1 What they are antagonistic towards is dimethlydiamminobenzene

EFFECT OF ANTIMETABOLITES OF DIMETHYL DIAMINOBENZENE ON SPONTANEOUS MAMMARY MOUSE TUMORS

TREATMENT	DAILY DOSE	ANIMALS	TRANSIENT REGRESSIONS	TRANSIENT OBLITERATIONS
	mg			
S ll		60	3	2
DMAP	3 5	30	6	5
CPA + DCDNS	2 0 2	14	5	2

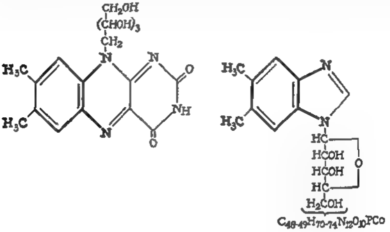
Tw f th g t w q ti bl

Fig 6

When either DMAP or a mixture of the two more complicated drugs CPA and DCDNS is injected into mice bearing spontaneous mammary cancers approximately half of the tumors decrease in size The data of

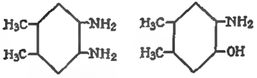
machinery of host and cancer and this difference is with respect to vitamin B_1 a highly active substance now considered to be concerned directly with the formation of desoxyribonucleic acids

Advantage can be taken of this difference with respect to vitamin B_1 between host and parasite to construct selectively toxic agents which will harm one but not both of the pair This has been done The substances involved are antimetabolites of dimethyldiaminobenzene This dimethyldiaminobenzene is the precursor from which living things make vitamin B_1 and riboflavin (Figures 3 and 4) Antimetabolites of it act to inhibit the



Riboflavin Vitamin B_{12}

FIG 3



Dimethyl-diaminobenzene DMAP

FIG 4

synthesis of vitamin B_{12} just as sulfanilamide inhibits folic acid formation Thus the tumor which synthesizes B_1 has the machinery which is susceptible to harm by these antimetabolites whereas the normal host tissues lack this machinery and hence escape the harm There is a little more to it than this but time will not permit us to explore it today In fact there is a very crucial point which will be found discussed in considerable detail in the papers previously referred to This point is how to avoid the so-called

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Suppression of Growth by Clinical Antibiotics

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At the present time there is no antibiotic available to the clinician that effectively suppresses virus growth This statement could well complete this portion of the symposium it is made however only to emphasize that none of the ever growing group of antibiotic substances so far introduced has proven effective on the smaller or as many would consider them the true viruses Diseases such as influenza smallpox poliomyelitis rabies yellow fever all remain a challenge to the experimental chemotherapist and a problem to the clinician

Nevertheless during the last few years a noteworthy breakthrough has occurred in the field of therapy We now have not one but a number of antibiotics that are highly effective in suppressing the growth of certain obligate intracellular parasites—the Rickettsiae and their small cousins the psittacosis group of micro organisms This is not the place for a discussion of taxonomy but it seems that these two groups of infectious agents deserve if not generic union at least recognition of their familial relationship However regardless of their final classification they grow within living cells hence they deserve consideration with the viruses Furthermore now that it has been demonstrated that the cell barrier is not insurmountable we can look forward to new antibiotics and synthetic chemicals that will strike directly at the infecting virus and ultimately many virus diseases will yield to specific therapy With this prospect in mind it might be well to review some of the methods employed in finding and evaluating new antiviral antibiotics and to discuss some of the pitfalls we have encountered in evaluating in the laboratory the presently available antibiotics

In reviewing the literature on this subject one cannot help but be impressed by two difficulties The first is the marked effect of experimental design on the results one obtains in the laboratory The second is the great

Figure 6 will illustrate this fact Some of the cancers continue to decrease until they are no longer palpable Although the data are not shown in Figure 6 a few of these obliterations persist as permanent cures in that no palpable tumor ever again is observed This occurs when the drugs are stopped when the tumor disappears If the drugs are continued all start to grow again after a variable period It is for this reason that one must speak of temporary obliterations In these cases one could say that the cancers have developed a drug resistance just as similar growths do towards every known oncostatic agent

If the development of drug resistance could be avoided and if all tumors could be made to regress and to disappear these agents would be promising compounds Even as they are they seem to be better than any known substance for suppression of *spontaneous* mammary cancers * Nevertheless if they do no more than to indicate to you that it is entirely possible to achieve selectively toxic compounds through considered use of antimetabolites I feel that they will have served a worthwhile purpose Sufficient delving in these quarters may uncover substances with practical usefulness

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* Some strains of transplanted cancers can be suppressed in a higher percentage of animals than is the case with the present substances However we are speaking here of *spontaneous* tumors rather than of transplanted single strains

schedules blood levels and toxicity that cannot be obtained in any other way For final evaluation one should if possible study therapy of the virus infection in several species because of differences in absorption conjugation etc factors which markedly affect the usefulness of any treatment

Regardless of the type of virus culture employed laboratory tests for antiviral activity fall into two main groups—screening and evaluating

The screening test is usually not elaborate in scope It employs few infected units whether they be eggs animals or tissue cultures and is designed as a relatively crude check for possible interesting activity The sensitivity is set moderately high and one plans to recheck all indications of possible activity no matter how slight This type of test with only minor modifications usually can be adapted to give a rough activity assay procedure for antibiotic isolation or analogue comparison It is the practice in our laboratory to use embryonated hen eggs exclusively in preliminary antiviral testing using only those viruses that kill the embryo within a rather narrow time range While this practice limits us somewhat in the selection of viruses it lends itself to uniformity and an adequate virus spectrum covering size range and tropism can be studied Of course susceptible laboratory animals usually mice or tissue culture tubes are being employed very adequately in other laboratories The final decision on the type of screening tests to be used depends on the virus diseases in which one is primarily interested the facilities available in the laboratory and the number of unknowns to be tested The design of the program is immaterial as long as a true antiviral chemotherapeutic effect is determined By this is meant that the infection should be established before treatment is started

At this point it might be in order to enter a plea for the organized screening program Such programs have been criticized as being an unscientific approach in that one makes no *a priori* hypothesis on essential metabolites or mode of viral reproduction but rather searches aimlessly using a shotgun rather than a rifle Also it has been stated that if the effort and money expended on screening programs instead were applied to fundamental studies of cell metabolism and virus cell interaction a reasonable and logical foundation for viral chemotherapy would soon be available The principle is fine but look at the record With the possible exception of para amino benzoic acid in rickettsial diseases all of the clinically useful chemotherapeutic agents that are now available were either bequeathed to us from the empirical lore of the witch doctor such as quinine or discovered through discerning but accidental observation by a trained scientist for example penicillin or they were developed from leads picked up in a screening program atabrine streptomycin chloramphenicol aureomycin isoniazid are but a few Fundamentally screening programs are nothing more than an organized method of increasing one s chances of making a lucky observation The fundamental approach has its place and is being followed by many but it is probable that the screening program will yield the clinically

difficulty in extrapolating laboratory results to the clinic These two problems are particularly apparent when an attempt is made to compare two antibiotics in the laboratory and then predict which will be the more effective clinically It must be concluded that even the best conducted laboratory evaluation is merely a qualitative indication of possible clinical effectiveness and that final evaluation must await careful clinical trials

Methods Used for Testing for Antiviral Activity

There are many methods of evaluating potential antibiotics in experimental virus diseases in the laboratory The procedures most commonly employed make use of the developing chick embryo susceptible laboratory animals and more recently tissue cultures

Fertile hen eggs when they can be utilized provide a convenient medium for the study of antiviral agents The effects on viral growth can be determined by (a) Inhibition of the development of macroscopic lesions on the chorio-allantoic membrane or in other tissues (b) Delay in mean death time of the embryos or an increase in number surviving (c) The depression of virus activity as evidenced by microscopic identification of virus inclusion bodies or elementary bodies (d) Serologic estimation of the quantity of virus antigen developed during incubation either by complement fixation hemagglutination or infectivity The route of infection depends somewhat on the virus being studied but treatment can be administered in a number of ways Intravenous therapy is possible but is rather laborious and similar results can be obtained by injection into the allantoic sac a route somewhat analogous to intraperitoneal treatment in animals The yolk sac route in the egg would correspond to oral administration and deposition between the chorio allantoic and white membranes gives a moderate repository effect similar in many respects to a subcutaneous injection

Tissue cultures provide a means for evaluation of antiviral agents under semi *in vitro* conditions and are being used more and more as methods are developed With this medium one is working in a relatively simple system and the influence of detoxification excretion and permeability is reduced to a minimum Also one can set the sensitivity at a very high level picking up leads that might have been missed by other methods For fundamental studies on mode of action these factors are of course a decided advantage *but for adequate evaluation one must eventually study the intact infected* animal

Experimental results obtained from the susceptible laboratory animal are generally based on death of the animal macroscopic lesions in the various organs and/or microscopic examination of the tissue for inclusion bodies and cell pathology For a number of diseases pathogenesis and the clinical disease can be closely approximated for example the infection of mice with rabies virus by intramuscular injection However even if this ideal is not possible animal infections supply information on routes of therapy dosage

viruses or the pox group There have been reports of clinical results or of apparent suppression of growth in the laboratory but the weight of the evidence is definitely that they are without effect

In our early experiments with Chloromycetin we found a reproducible and significant prolongation of the life of mice infected with various strains of influenza virus[9] Similar results were obtained in chicks infected with the virus of Newcastle disease However in repeated experiments in chick embryos we could demonstrate no inhibiting effect of chloramphenicol on the growth of these viruses Subsequent work has demonstrated that it is very probable that this prolongation of life in animals infected with these viruses is due solely to suppression of secondary bacterial invaders rather than to any direct antiviral effects This experience is mentioned here as it is possible that others may be similarly misled in the interpretation of animal antiviral tests with highly potent antibacterial substances

None of these broad spectrum antibiotics at the concentrations developed in therapy inactivate the micro-organisms on which they are effective They act by suppressing multiplication not by killing the invading organisms This has been demonstrated repeatedly in the laboratory and it has a broad implication in the planning of a therapeutic regimen Since these antibiotics only suppress growth one must depend on the natural defenses of the host to eradicate the infection If these defenses have not been alerted by the early stages of the infection or if they do not operate properly a relapse will usually occur after cessation of therapy This is the rationale for the combined use of typhoid vaccine and Chloromycetin in the treatment of typhoid fever—or as Woodward[10] has suggested for the deliberate withholding of antibiotic therapy in early cases of rickettsial and virus diseases until the defenses of the patient have been stimulated Dr Woodward who is next on this program will probably have more to say on the clinical importance of this aspect of antibiotic therapy

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effective antimetabolite and that only then will be discovered the unsuspected essential viral metabolite with which it interferes Advocating the screening program does not mean that we cannot be scientific rather we should tax our ingenuity to the utmost to devise the best laboratory models of diseases for evaluating our leads Antiviral leads whether developed empirically or by deduction are few and far between and deserve careful and well planned study

The evaluating tests to be applied to a promising lead cannot be defined It is in this phase of experimental chemotherapy that the investigator has the greatest difficulty There are a number of questions however that one should try to answer What is the mode of action? It may be a direct virucidal effect on the infecting elementary bodies It may be a suppressing effect on some phase of the virus cycle If so where—and is it on the virus or on the host cell? What is the toxicity activity ratio? Can animals in which symptoms have developed be saved or might the drug be used prophylactically? These and many other questions arise but if one bears in mind how the drug can be applied in human disease it is usually possible to decide if a clinical trial is warranted As stated before final evaluation must come from the clinical case but the laboratory can and should provide data on safety dosage schedule route of therapy and some evidence of possible clinical usefulness before such tests are indicated

Laboratory Results with Clinical Antibiotics

The clinical antibiotic that first showed any suppressive effect against a virus was penicillin It was found to inhibit the growth of murine feline and meningopneumonitis by Eaton and his co workers [1] Also in studies with the pox group Groupe and Rake reported that a number of these agents were inhibited by relatively crude preparations of commercial penicillin This activity was traced to an impurity in the preparation used pure crystalline penicillin being without effect It is likely however that the impurity was also an antibiotic Streptomycin the next antibiotic to be introduced was found by Smadel s group[3] to show some slight inhibitory effect on a number of Rickettsiae when very large doses were used However neither penicillin nor streptomycin has proved to be of any value in the primary treatment of virus diseases even though they have been widely used to control secondary bacterial invaders

With the advent of chloramphenicol[4] and aureomycin [5] the era of the so-called broad spectrum antibiotics was opened These drugs have proved to be truly remarkable chemotherapeutic agents While their range of usefulness is not as broad as some had hoped they have proven highly effective in controlling many bacterial infections as well as the rickettsial diseases and infections caused by the psittacosis—L G V group of viruses These antibiotics and others that have since been introduced—terramycin [6] erythromycin [7] and magnamycin[8]—have no direct effect on the growth of the small

viruses of the pox group There have been reports of clinical results or of apparent suppression of growth in the laboratory but the weight of the evidence is definitely that they are without effect

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Clinical Application and Mode of Action of Antibiotics in Rickettsial and Virus Diseases

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Introduction

Specific chemotherapy has been achieved for most of the bacterial diseases and during the last five years has been extended to rickettsial and viral diseases It is of historical interest that our earliest specific remedies—quinine mercury antimony and the salvarsans—were effective chiefly against the larger infectious agents the protozoa and the spirochetes With the introduction of sulfonamides the bacteria particularly the gram positive microorganisms became vulnerable The antibiotics with penicillin in the forefront further extended the field of specific medication without however showing much effect upon the diseases caused by gram negative bacilli Later certain gram negative microbes yielded to streptomycin s action Yet it is well known that its effect in the group of still smaller infectious agents—the rickettsiae and viruses—is of no clinical significance Three broad spectrum antibiotics chloramphenicol aureomycin and terramycin [1 3] not only have combated many important human infections caused by gram positive and gram negative microorganisms but have shown themselves to be highly specific for all members of the rickettsial group Furthermore they have significant effect on a few viral agents of disease

The therapy of viral diseases has merely been broached and future advancements undoubtedly will pertain to this group of microbes Major emphasis in this report therefore will be placed upon the rickettsiae However those viral diseases susceptible to specific medication also will be discussed

Application of the broad spectrum antibiotics to the rickettsioses has provided the therapist with a practical greatly simplified treatment which has reduced mortality virtually to zero

Chloramphenicol aureomycin and terramycin were shown in 1947 1948 and 1950 respectively to possess potent anti rickettsial properties [4,5,6,7] Pharmacological investigations in animals and humans established that these drugs could be reasonably well tolerated orally in dosages sufficient to produce measurable blood concentrations and to inhibit rickettsial activity

Initial clinical trials showed the merit of chloramphenicol in patients ill with epidemic and murine typhus [8,9] These trials were soon supplemented by dramatic results with this antibiotic in scrub typhus [10] Rocky Mountain spotted fever [11] and murine typhus [1] Simultaneously the effectiveness of aureomycin was demonstrated in typhus fever [13] Rocky Mountain spotted fever [14] and Q fever [15] The efficacy of terramycin in these diseases was reported later [16,17,18,19] Rickettsialpox was shown also to yield to this antibiotic therapy [0] Investigations abroad demonstrated that patients with *fievre boutonneuse and South African tick bite fever responded in a uni*formly favorable pattern [1,27] There are no published instances in which the human rickettsioses have failed to respond to broad spectrum antibiotic therapy provided the patient is given a reasonable chance for recovery

Dosage and Toxicity

Based upon extensive clinical trials the following dosage schedule is considered optimal for these antibiotics For chloramphenicol an initial oral dose of approximately 50 mg /kilogram of body weight is given while the dose for aureomycin and terramycin is based on 25 mg /kilogram of body weight Subsequent maintenance doses are calculated on the basis of 50 mg /kilogram per day for chloramphenicol and for aureomycin and terramycin 25 mg /kilogram per day dividing the daily requirement equally and giving it at 6 to 8 hour intervals Antibiotic therapy is continued until the patient has improved and has been afebrile for approximately 24 hours When the preferred oral route is not feasible these antibiotics may be given intravenously observing the following dosages as the initial dose chloramphenicol 20 mg /kilogram aureomycin and terramycin 5–10 mg /kilogram of body weight and subsequent daily doses calculated in the same manner but administered in equal amounts at 6 hour intervals These schedules have been employed extensively in most of the rickettsioses and represent unless otherwise stated the regimens used in managing patients described in this report

Broad spectrum antibiotic therapy has been attended by certain undesirable side effects Nausea vomiting glossitis diarrhea and proctitis have been encountered more commonly with aureomycin and terramycin than with chloramphenicol Long term administration of any of the three broad spectrum antibiotics has been associated with various superimposed infec

tions including moniliasis Such sequelae have been uncommon following the short term therapeutic regimens required for treatment of the rickettsioses

Certain disorders of the hemopoietic system including aplastic anemia [8 29 30 3] thrombocytopenia [31] and pancytopenia [31] have been ascribed to chloramphenicol These dyscrasias have usually been reported in patients receiving the antibiotic over long periods [8 9 30] and rarely in patients on shorter regimens Such serious complications have been uncommon or rare in general practice There are no reports of adverse hemopoietic reactions following antibiotic therapy of patients with rickettsial diseases One report concludes that aplastic anemia is no more frequent following chloramphenicol than following other broad spectrum antibiotics [32] Hence the decision to use broad spectrum antibiotics in rickettsial diseases should not be influenced or weighted by fear of blood dyscrasia

Clinical Application

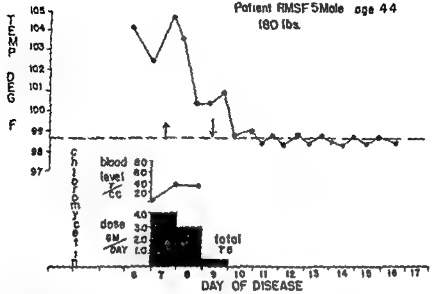
In the United States Rocky Mountain spotted fever provides the best model for demonstrating the effectiveness of antibiotics in rickettsial diseases The disease widespread throughout this country and Latin America is one of the more virulent rickettsioses simulating epidemic typhus in duration severity complications and mortality

Course of Illness in Moderately Ill Patients The dramatic impact of chloramphenicol on the clinical course of a patient with Rocky Mountain spotted fever is illustrated graphically in Figure 1

This 44 year old man was hospitalized on the 6th day of illness with a history of protracted frontal headache pyrexia of 102 F malaise weakness and a body rash Examination revealed injected and suffused conjunctivae and a non fixed pink macular rash over the entire body exclusive of the hands face and neck His cough was productive of blood tinged sputum Chloramphenicol was started on the 7th febrile day and continued until the 9th day A total of 7 5 g was given Headache abated within 24 hours the exanthem faded in 2 days and the temperature reached and remained at normal levels on the 9th day Clinical diagnosis of Rocky Mountain spotted fever was confirmed by isolation of *Rickettsia rickettsiae* from the patient s blood on the 7th day of disease and by significant antibody titers demonstrated by complement fixation and Weil Felix tests

This clinical record was selected because it typifies the pattern of response in moderately ill patients who are treated with any of the broad spectrum antibiotics It may be seen that specific therapy was safely discontinued at the time of defervescence

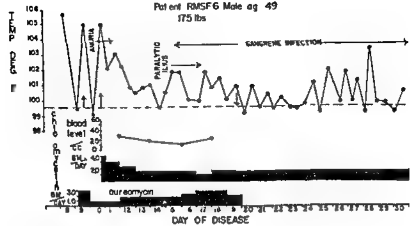
Course of Illness in Critically Ill Patients This 49 year old colored man was admitted in a near comatose state on the 8th day of illness Little was known of his illness prior to hospitalization except that it was characterized by intense headache fever for about a week and mental confusion shortly



Rickettsiemia	+
Complement fixation	1/32
Proteus ox 19	C 1/80 P 1/160 1/1280
WBC thousand	7.05

FIG 1 Rocky Mountain Spotted Fever Course of illness in a moderately ill patient treated with chloramphenicol Temperature reached normal levels in 2.5 days (Reprinted from *Am J Med* 3 308 1950)

before admission When first seen the patient was confused, irritable, and dehydrated Examination showed a rapid pulse blood pressure 90/70 tachypnea and various neurological alterations as spasticity muttering delirium meningismus and continued purposeless movements of the extremities When the circulatory status was stabilized by the administration of intravenous fluids a purpuric hemorrhagic rash with diffuse coalescing lesions over the extremities and trunk was noted Aureomycin therapy was instituted on the 9th day of disease and was supplemented on the 10th day with chloramphenicol During the early stage of treatment in order to combat the hypochloremia hypoproteinemia anemia and azotemia energetic supportive measures included transfusions of plasma saline glucose and whole blood Electrolytic requirements were gauged by frequent laboratory and clinical evaluations Constant nursing care was given and a liquid diet providing approximately 2 g of protein per kilogram of body weight per day was administered by stomach tube and later by oral feedings After 24 hours of treatment there was slight improvement and after 36 hours it was apparent that the patient would survive The skin lesions did not regress

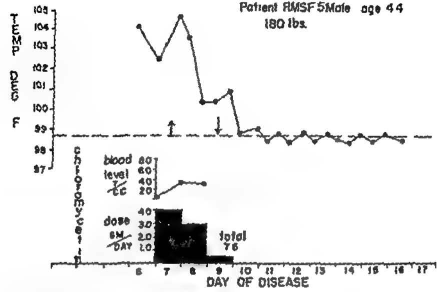


R ckettsiemia	+ 0
complement fixation	0 0
Proteus ox 19	1/40 1/640 [illegible] 49th DAY
W.B.C. thousands	12.40 17.93 17.30 13.05
urea N mg/100cc	96 116 166 128 134 130 408 83 15 12
blood pressure s/d	[illegible] / 70 64 0 50 83 85 70 54 70 76 65 70

Fig 2 Rocky Mountain Spotted Fever Course of illness in a critically ill patient with manifestations of skin ecchymosis peripheral vascular weakness marked azotemia and coma Treatment with two suppressive antibiotics Recovery gradual but complete Diagnosis confirmed by isolation of rickettsiae from blood and by positive Weil Felix reaction

but became gangrenous and ultimately sloughed to form superficial scars One extensive lesion of the right ankle progressed into a large area of tissue gangrene and local osteomyelitis A skin graft was applied to the leg Nevertheless the patient slowly and progressively improved and was discharged without significant residual effects Figure 2 is a graphic presentation of the pertinent clinical and laboratory findings

This patient was selected for presentation because he exemplifies the clinical course of those first diagnosed late in the course of illness Therapeutically the problem is one of specific therapy integrated with intelligent supportive care In this instance two suppressive antibiotics were utilized because when this patient was treated the severity of illness was thought to require heroic measures It is now known that single antibiotic therapy is adequate for all the rickettsioses Of additional significance is the fact that after 14 months convalescence viable organisms identified as *R rickettsiae* were isolated from this patient s excised lymph node This observation demonstrated conclusively that the rickettsiae of Rocky Mountain spotted fever like *Rickettsia orientalis* [33, 34] may persist in the tissues of recovered



Rickettsiemia	+		
Complement fixation			1/32
Proteus ox 19	CT 80 PI 160	1/1280	
W.B.C. thousands	7.05		

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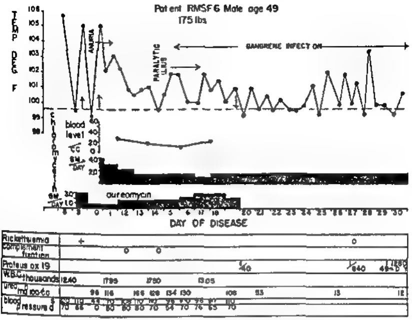

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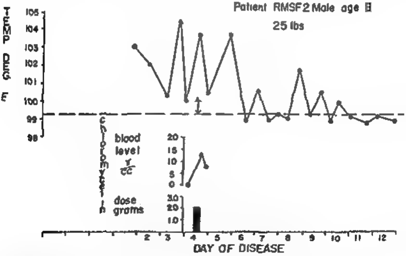
patients for variable periods of time Later these findings will be discussed in relation to immune factors and the tendency to relapse

Single Dose Treatment of Rocky Mountain Spotted Fever Smadel and his group have shown that scrub typhus may be treated effectively with a single large dose of chloramphenicol Figure 3 below is a graphic presentation of the significant findings in a patient with Rocky Mountain spotted fever treated with a single 2 0 g dose of chloramphenicol on the 4th day of illness Although the principal manifestations of the disease were ameliorated within 36 hours after the antibiotic was administered the temperature did not reach normal levels until 7 days after therapy The further course was uneventful

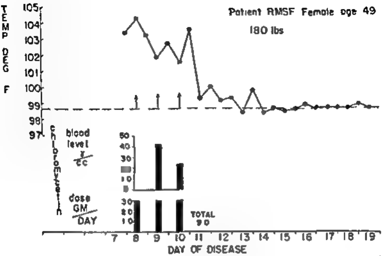
Two other patients were treated in the same manner with similar findings each showing intermittent febrile courses for approximately 7 days Although this regimen is not recommended the findings provide additional information as to the minimal effective dose in this rickettsial disease

Payne et al[8] demonstrated that epidemic typhus could be treated with a single daily dose of chloramphenicol Our group has employed a single daily dose of 3 0 g for 3 consecutive days Three patients with Rocky Mountain spotted fever were treated in this manner Three hours after ad



Rickettsiemia	+	
complement fixation	0	0
Proteus ox 19	0	1 1280
WBC thousands	90	70

Fig 3 Rocky Mountain Spotted Fever Clinical course in a mild case treated with a single dose of chloramphenicol Patient was free of headache and toxic manifestations other than fever in 48 hours Convalescence uneventful (Reprinted from *Am J Med* 3 308 1950)



Rickettsiemia	+ 0 0
complement fixation	0 0 1:40 36 DAY
Proteus ox 19	1/40 1/160 1:160 36 DAY
WBC thousands	90

Fig 4 Rocky Mountain Spotted Fever Clinical response in an adult female treated with single large daily doses of chloramphenicol (Reprinted from *Am J Med* 3 308 1950)

ministration of the drug serum concentrations of the antibiotic ranged from 14 to 48 gamma/ml The clinical course of one of these patients is graphically presented in Figure 4 It is noted that the febrile course is slightly delayed when compared with the results obtained in patients treated by conventional regimens

Modification of the Clinical Course with Cortisone The persistence of fever for 2 to 4 days following antibiotic therapy the development of shock and peripheral vascular weakness and the continuation of headache and other manifestations of toxemia for variable periods of time prompted a search for ancillary measures Utilization of adrenal cortical hormones as anti toxic agents in bacterial infections has been frequently reported [35 36 37] and it is known that the course of scrub typhus may be significantly shortened by the concurrent administration of antibiotic and cortisone acetate [38] Various European investigators have empirically employed adrenal hormones in rickettsial infections with encouraging results It has been surmised that in severely ill patients a prolonged period of stress coupled with possible toxic adrenal damage and depletion of metabolites essential to the adrenal cortex might lead to an insufficient production of adrenal cortical hormones [39]

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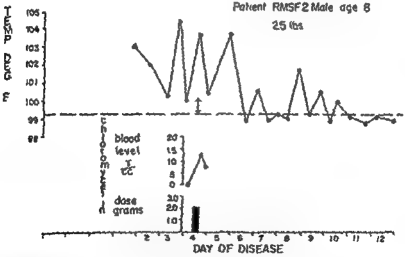

Rickettsiemia	+		
complement fixation	0	0	
Proteus ox 19	0		1 1280
WBC thousands	90		70

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and toxic manifestations of scrub typhus[38] and Rocky Mountain spotted fever[40] may seem of theoretical interest only but it is of practical value in patients first observed late in the course of their illness when supplemental anti toxic measures may be desired

Summary of Results of Treatment of Rocky Mountain Spotted Fever

Our experience with aureomycin and terramycin has been less extensive than with chloramphenicol Nevertheless our findings have been similar to those of others and indicate that the therapeutic results with these two antibiotics parallel those with chloramphenicol A summary of our experience with antibiotics in the treatment of Rocky Mountain spotted fever since 1948 is presented in Table 1

Table 1

TREATMENT OF ROCKY MOUNTAIN SPOTTED FEVER 1930–1953

Drug	*No*	*Mean Day Disease ℞ Started*	*Mean Duration Fever After ℞ Started*	*Percent Fatality*
Chloramphenicol	37	6 1	3 1	[illegible]
Terramycin	6	6 4	2.5	0
Aureomycin	2	6 0	3 1	0
Chloramphenicol Cortisone	14	6 4	1 8	0
Untreated 1930–1945	85		16 5	23 5

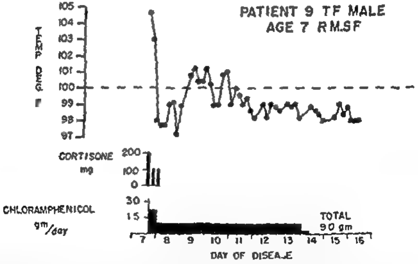
Thirty seven patients with Rocky Mountain spotted fever treated with chloramphenicol experienced defervescence in an average period of 3 1 days after institution of therapy Mean values for 6 patients receiving terramycin and 2 patients given aureomycin was 2 5 and 3 1 days respectively Therapeutic benefits obtained with the three antibiotics are not amenable to comparison because of the disparity of numbers in each group The speed with which the fever abated in the chloramphenicol series compares with the results obtained by Ross et al with aureomycin [14] and Powell and others who employed terramycin [illegible] All these antibiotics convert an extremely sick and toxic patient [illegible] one who is comfortable and afebrile in approximately 72 hours These dramatic results leave little to be desired in [illegible] disease which formerly showed in 85 cases carefully studied in our hospital an average duration of fever of 16 5 days an average hospitalization of 22 0 days and a fatality rate of 23 5%[1]

Noteworthy in our experience is the reversability of the illness regardless of the severity or stage at which treatment is started It naturally follows that the temperature response is less dramatic in patients with advanced changes including extensive hemorrhages of the skin and parenchymal tissues Nevertheless with intelligent antibiotic and supportive treatment

Our group treated 14 patients on a combined regimen of chloramphenicol and cortisone Adult patients received an initial dose of 200 mg followed by two doses of 100 mg of cortisone at 6 hour intervals Children received two-thirds the adult dose In this group therapy was instituted on the 3rd to 13th day of disease with a mean of 6 4 days In each case response was excellent including three seriously ill patients in whom therapy was instituted late in the course of their disease The most striking observation was allevia tion of headache dissipation of the toxic state and return of appetite The duration of fever after the institution of combined therapy averaged 1 8 days

Figure 5 is a graphic presentation of the course of illness in a 9 year old boy severely ill with Rocky Mountain spotted fever treated with this combination on the 7th day of disease Eight hours after institution of treatment the temperature had reached normal levels and the nausea vomiting and headache had completely abated All toxic manifestations were ameliorated in 24 hours and except for a transient slight temperature elevation following the release from cortisone effect the future course was uneventful

The fact that hormone therapy exerts an ameliorating effect on the febrile



RICKETTSIEMIA		0 0	
WEIL FELIX	OX 19	1:1280	1:1280
	OX 2	1:20	1:20
COMPLEMENT FIXATION		1:20	1:40
WBC (thousands)		9 8 7 5	

FIG 5 Rocky Mountain Spotted Fever Clinical course in a severely ill boy treated with combined chloramphenicol and cortisone Note temporary low grade fever representing release from cortisone effect Diagnosis confirmed serologically (Reprinted from *New Eng J Med* 246 962 1952)

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toxemia is ameliorated circulatory hepatic and renal damage gradually regresses and recovery is the rule

Fourteen patients treated with both chloramphenicol and cortisone at varying stages of illness (mean 6 4 days) experienced more rapid dissipation of the clinical manifestations of the disease The average value for duration of fever in this group was 1 ▮ days Several patients with advanced disease unduly lengthened the average values for temperature response and in many the total febrile and toxic period was approximately 12 hours These findings are primarily of academic import although the addition of cortisone to the therapeutic regimen in the late advanced case has its place

Summary of Results in the Major Rickettsioses

Each of the three broad spectrum antibiotics has been used extensively in the therapy of human rickettsial diseases Twenty six patients with classical louse borne typhus have been treated with chloramphenicol by Payne[8] and Smadel[9] 4 with aureomycin by Fu[41] and 32 with terramycin by Knight Ruiz Sanchez and others[13 16 17] The length of the febrile period following institution of therapy averaged 2 0 days for chloramphenicol 3 0 for aureomycin and 4 0 for terramycin

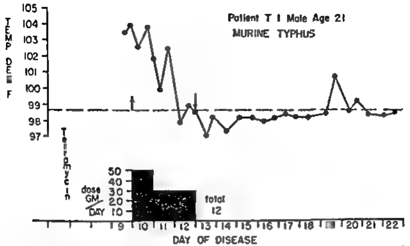
Chloramphenicol has been used in recrudescent typhus or Brill s disease and produced defervescence in approximately 4 days in two patients treated by Murray et al[4] and Knight et al[43] Aureomycin produced defervescence in two days in four additional patients[44 4 46]

The clinical features of flea borne or murine typhus fever are similar to but less severe than those of the classical type Without specific therapy the anticipated febrile course in murine typhus ranges from 9 to 15 days and the fatality rate is less than 2% In 64 aureomycin treated patients reported by various authors the temperature reached normal levels in 48 hours[13 43 47 45] Ley and his colleagues[30] and Ruiz Sanchez[47] treated 17 cases with chloramphenicol these became afebrile within 3 days after beginning therapy In 5 patients treated with terramycin[13 43] a similar response was noted

Shown in Figure 6 below is the graphic record of a 21 year old man with murine typhus who responded satisfactorily to terramycin although therapy was first given on the 9th day of disease Rickettsiemia was demonstrated just prior to institution of treatment Slow and steady progress was made and temperature reached normal levels on the 12th day

Rickettsialpox a mild form of rickettsial infection antigenically related to Rocky Mountain spotted fever has responded very satisfactorily to antibiotic therapy Twenty five patients treated by Rose[ʻ] rapidly improved with defervescence occurring within 2 days after initiation of specific treatment In this series 9 patients received aureomycin 8 chloramphenicol and 8 terramycin One patient treated with streptomycin failed to respond

Q fever is now widely recognized in Europe Australia and the United States as a disease capable of occurring in epidemic proportions Evidence



		9	12	14	16	19	21
Rickettsemia		+					
Proteus ox19		1/40		1/640		1/640	1/640
WBC thousands			8.1				
CF	Murine	0		0	0	0	1/20
	Epidemic	0		[illegible]	0	0	0

FIG 6 Murine Typhus Course of illness in a moderately ill patient treated with terramycin Diagnosis confirmed by isolation of *Rickettsia mooseri* from blood and by serologic tests (Reprinted from *Ann N Y Acad Sci* 53 395 1950)

has accumulated rapidly during the past several years which indicates that all three broad spectrum antibiotics are useful in the treatment of human infections Evaluation has been difficult because of the variability in severity and duration of this illness when untreated Aureomycin has had more extensive clinical use than chloramphenicol or terramycin Fifty two patients treated with aureomycin by Clark and Lennette[15] and Fellers[49] responded favorably with return of temperature to normal levels in 5 days One group of authors[15] were impressed with the variable response and reported relapses following cessation of specific therapy The rickettsiostatic properties of the antibiotic were considered to be a factor in relapse tendency Chloramphenicol s action has been shown to be therapeutically similar in 24 patients treated by various authors with defervescence in about 3 days [15 49 50 51] Seven patients treated with terramycin became afebrile in 3 days [5 53]

Scrub typhus emerged in World War II as a disease of major importance and ranked high as a cause of death from infections in military personnel In 1948 the U S Army Typhus Team working in Malaya in collaboration with British investigators determined that chloramphenicol was highly efficacious in the treatment of scrub typhus Ninety four patients treated with chloramphenicol experienced defervescence in an average of 30 hours fol

lowing institution of therapy These investigators noted similar dramatic findings in 30 patients treated with aureomycin and in 46 patients treated with terramycin Without exception the response to all these antibiotics was uniformly favorable and noteworthy in these studies is the reversability of the clinical condition even when therapy was first given late in the course of disease Recovery is rapid in scrub typhus *complications are negligible* and mortality is zero Shown in Figure 7 below is the clinical record of the first scrub typhus patient to receive antibiotic therapy This 26 year old soldier first treated on the 5th day of illness became afebrile in 30 hours All outward manifestations of illness abated within the first 24 hours and the patient rapidly regained strength and vigor

Relation of Mode of Action of Antibiotics and Immunity in Rickettsial Diseases

There can be no doubt that the dramatic beneficial effects in all rickett sioses are the result of the antibiotic s suppressive rather than rickettsiocidal action *Investigations in scrub typhus provide the best model for a discussion* of the relationship of these factors to mode of action and immunity

Immunity in most infections is dependent upon a mass of antigen suffi cient to stimulate an immune state The time necessary for provocation of this defense varies with the pathogenic agent and the host s response to the specific stimulus For example recovery from a natural typhoid infection of six weeks duration is attended by satisfactory immunity in 90% of in stances and clinical and bacteriological relapse in 10% The utilization of chloramphenicol in this disease results in more prompt amelioration of the clinical manifestations but here the occurrence of relapse is 20% or higher Immunity to scrub typhus and other rickettsial infections acquired under natural conditions is produced in shorter periods of time (i e two weeks) In the non fatal untreated case recovery occurs after approximately two weeks of illness and relapse is rare A notable exception is Brill s disease a recurrence of epidemic typhus This disease exemplifies the concept that *viable rickettsiae may remain for variable periods in the host in an essen* tially unaltered state Smadel and his collaborators have been successful in isolating *R orientalis* from chloramphenicol treated scrub typhus patients two years after recovery [33] Similarly [34] viable *R rickettsiae* have been isolated from *the lymph gland* of a patient one year *after recovery from* Rocky Mountain spotted fever

Naturally acquired scrub typhus is usually first diagnosed with the ap- pearance of the skin exanthem on the 5th to 7th day of illness The clinical response following antibiotic therapy is prompt Suppression of rickettsiae with chloramphenicol or other antibiotics for 24 or more hours is sufficient to cure and relapses are uncommon Similar findings are true in murine and epidemic typhus and Rocky Mountain spotted fever Contrariwise when therapy with a rickettsiostatic drug is instituted within the first several

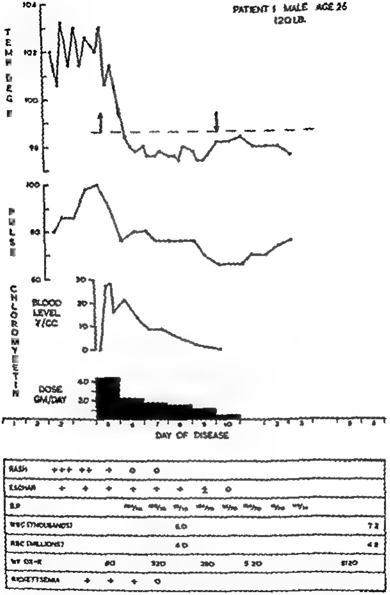


FIG 7 Scrub Typhus Clinical response in a patient to chloramphenicol therapy begun on the 5th day of disease A total of 11 6 g of chloramphenicol was given over a period of 120 hours (Reprinted from *J Cl Invest* 28 1196 1949)

days of disease clinical suppression of the illness is rapidly manifest but relapse frequently occurs after the antibiotic is discontinued [54] Moreover continued rickettsiostasis by the daily administration of chloramphenicol first administered simultaneously with infection merely serves to prevent multiplication of rickettsiae and active disease then ensues on cessation of drug treatment [4] These factors may be demonstrated graphically in Figure 8

Under Situation A the usual pattern reveals that relapses did not occur when specific therapy began around the 6th day of disease regardless of the duration of treatment On the other hand when therapy began quite early such as the second febrile day relapse was the rule about 5 days after treatment was discontinued Situation C is a graphic presentation of the findings in a human trial in which continued rickettsiostasis was maintained 28 days after an artificially induced infection Suppressive antibiosis for 28 days was insufficient to allow time for immunity to develop and full blown active disease occurred in this group [4]

Eventual immunity is dependent upon paying the piper to a certain extent In Situation B it is readily noted that the intermittent administration of chloramphenicol at 7 day intervals in naturally infected human volunteers did permit the host to develop his own immune defenses Continuation of these intermittent courses up to the 35th day did prevent the disease from appearing in the active sense However the mild headache and

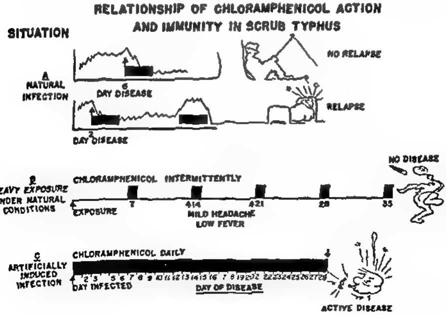


Fig 8 Relationship of Chloramphenicol Action and Immunity in Scrub Typhus

low grade fever noted just prior to several of the intermittent doses were undoubtedly manifestations of infection At these times *R orientalis* is readily isolated from the patient s blood By further adjusting the dose of antibiotic and treatment interval (i e 4 day intervals up to the 25th day) it is possible to completely suppress the clinical disease Rickettsiema may be demonstrated and immunity subsequently develops Immunity therefore comes at a price dependent in large part upon the mass of antigenic stimulation and resultant immune response

The isolation of viable rickettsiae several years after active infection from scrub typhus and Rocky Mountain spotted fever patients who received rickettsiostatic antibiotics lends further confirmation that the mode of action of these antibiotics is suppressive rather than killing The occurrence in animals of inapparent infections and the evidence that viable rickettsiae may remain in convalescent scrub typhus and Rocky Mountain spotted fever patients point to the possibility that latent forms of all the rickettsioses besides those of the Brill s type may occur

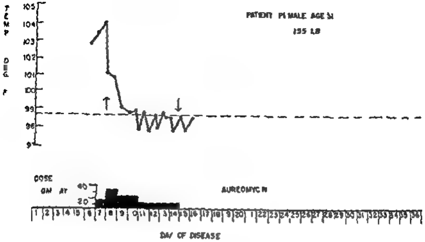
Clinical Applications in Viral Infections

Modern antibiotic therapy has enabled the physician to attack certain of the larger viral agents directly rather than to rely solely upon the older method of symptomatic care The psittacosis lymphogranuloma group of agents are intermediate in relation to the small viruses and rickettsiae and possess certain clinical and biological characteristics of each

Penicillin is not devoid of therapeutic efficacy in patients ill with psittacosis[5] and lymphogranuloma venereum[64 66] Nevertheless its value is limited and unless penicillin is administered extremely early in the disease its contribution is essentially one of controlling the secondary infection Each of the three broad spectrum antibiotics showed value against the psittacosis lymphogranuloma viruses when tested in the laboratory and fulfillment of these promises are slowly being accumulated clinically[57 8 59 61 62 63 64 6 67 68]

Shown below in Figure 9 is the graphic record of a patient ill with ornithosis successfully treated with aureomycin This 51 year old man first received aureomycin on the 7th day of an illness characterized by severe headache fever cough and a bilateral pneumonitis Recovery was prompt and convalescence uneventful Diagnosis of a psittacosis infection was confirmed by demonstration of a rise in titer of complement fixing antibodies One week prior to illness the patient had trapped 80 pigeons in the rafters of his barn

This case is presented to demonstrate the usual clinical response in patients with psittacosis or ornithosis who are treated with the broad spectrum antibiotics In many instances the clinical manifestations mimic those of primary atypical pneumonia although the therapeutic results are more uniformly favorable in psittacosis



W B C	10,850 9400 7650		
COLD AGGLUTININS	0	0	0
PSITTACOSIS C F	1/16	1/16	1/[illegible]
Q FEVER C F	0	0	0

FIG 9 Ornithosis Course of illness in a patient successfully treated with *aureomycin Infection acquired from pigeons* and characterized by *pneumonitis* headache and *toxemia* Convalescence rapid and uneventful Diagnosis confirmed by serologic methods (*Reprinted from Ann Int Med* 31 53 1950)

Penicillin is of limited value in treatment of patients with lymphogranuloma venereum whereas the new antibiotics are of value in lymphogranuloma during the primary stage and against the secondary invader in the chronic form The need for surgical correction of anatomical alterations of tissue has not been obviated

Progress has been made chemotherapeutically in treatment of trachoma which is caused by a virus similar to that of the psittacosis lymphogranuloma venereum group Penicillin given systemically or locally has been helpful[69 70] Chloramphenicol[72 72] aureomycin and terramycin[73 74] have demonstrated their effectiveness in the treatment of the acute form of trachoma and in the chronic form to a lesser extent provided therapy is prolonged for several months Acute pannus and corneal ulceration have been cured in several days *with application of terramycin or aureomycin*[73 74] Inclusion blennorrhea is an acute form of viral conjunctivitis which is common in the newborn as well as in adults Sulfonamides[75] penicillin[76] and aureomycin[77] applied locally and systemically are effective

There are a group of viral *infections in which the chemotherapeutic* and antibiotic agents exert no direct action upon the causative agent per se but rather benefit the patient through the suppression of secondary or super

imposed bacterial infections Antibiotic therapy of measles variola varicella epidemic influenza and pemphigus are examples Indirect helpful action may be observed in cases of Herpes zoster infectious hepatitis and epidemic parotitis where there is no known anti viral action of the antibiotic

Primary atypical pneumonia a disease of protean clinical and pulmonary manifestations is loosely called virus pneumonia although no virus has been definitely identified as the etiological agent in the majority of cases Treatment prior to the use of the broad spectrum antibiotics was mainly symptomatic With currently available drugs it is generally accepted that aureomycin [78] terramycin [79] and chloramphenicol [80,81] alleviate the acute manifestations and shorten the febrile course Infiltration in the lung as shown by roentgen examination is little altered by these antibiotics and the disease tends to run a more protracted course than pneumococcal lobar pneumonia

Although recent years have witnessed a partial conquest of the viruses by chemotherapeutic agents continued investigations in this field make it reasonable to predict the eventual inclusion of the viruses into the realm of diseases for which there is specific treatment

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DISCUSSION

Approaches to Prophylaxis and Therapy of Virus and Rickettsial Infections

DR DALLDORF (Moderator) Doctor Rasmussen uses the occurrence of paralysis as his criterion rather than death or residual paralysis which are the important criteria of human disease It might be interesting if some of his monkeys survive to see whether they recover Years ago when we were investigating the sparing effect of lymphocytic choriomeningitis on experimental poliomyelitis we frequently had groups of animals that were so little protected they became completely prostrated were completely paralyzed We nursed some of them for a few days and when they recovered enough to feed themselves we put them into a common pen I was greatly surprised some weeks later to see that almost all had recovered completely Our controls usually died no matter how well we nursed them

I mention this not only to explain why I would be interested in the final results of Doctor Rasmussen s experiments but also to venture the opinion that poliomyelitis may be more amenable to treatment than we suspect It is always difficult to judge the susceptibility of a morbid process to treatment until we have a treatment that has some effect Lobar pneumonia and subacute bacterial endocarditis seemed quite as hopeless therapeutically as poliomyelitis does today Yet there is reason to suspect poliomyelitis is reversible and that even moderation of the disease phenomena could tip the scales in a way that would have great practical benefits

DR J W CZEKALOWSKI (Univ of Leeds England) It is a privilege to contribute to the discussion on the excellent presentations given to us today by Drs Horsfall and Woolley The work on blocking and physico chemical interference was undertaken undoubtedly with a thought of finding chemical compounds which could be applied eventually in the chemotherapy of viral diseases

In an endeavor to approach the same object we tried the effect of various known enzyme inhibitors on the reproduction of phages Bacterium phage system (*E coli* strain B and T2 phage) was chosen because in some respects it is quite a unique experimental system yielding data which could be quantitatively assessed with great accuracy Now let me summarize briefly some work done in the past

We share Dr Woolley s view that the ideal active substances should not affect the host cells in any way and yet should interfere with the reproduction of the viral agent

The chemical substances examined* could be divided into 5 groups ac

* Nature London (1949) 163 719 *Brit J Exp Path* (1952) 33 57

cording to their action on the constituents of the experimental system namely the host cells and the phages I should like to confine myself only to two groups of substances which have in common the property of not affecting the course of reproduction of the host cells but differ in respect of their action on the bacterial viruses one group has a static effect and the other a destructive one on the phage T2 These active substances belong to many chemical groups but they seem to share in common the property of interfering with the enzymes which work in conjunction with the cytochrome system and especially that of interfering with the succine oxidase system It is well known that inhibitors of this system may act either specifically or non specifically and it seems that most of the substances investigated by us belong to the group acting in the former fashion

Among the active substances known to inhibit the sulphydryl groups con taining enzymes—to which succinic dehydrogenase belongs—I should men tion urethane selenite mapharside colchicine (though it may affect also other enzymes) and also auramine and tetra–methyl–*p*–phenylendiamine All these substances inhibited virus growth but malonic acid the most specific inhibitor of succinic dehydrogenase in the isolated experimental enzyme systems did not (probably by virtue of its failure to penetrate into the intact E coli cells)

The *p*–phenylendiamine hydroquinone phenylurethane and BAL may act on the so called respiratory catalyst a link between cytochromes b and c Naphthoquinones act on some factor which is neither cytochrome b nor c nevertheless it prevents the interaction of these two elements

Among the inhibitors acting on the other parts of the cytochrome system we should mention cyanide which inhibits cytochrome oxidase and fluoride which acts probably nonspecifically by virtue of its affinity to heamatin compounds

It is difficult if permissible at all to draw any comparison between the cytochrome systems of mammalian cells and of micro-organisms in view of qualitative differences between them The cytochromes of *E coli* grown aerobically differ from their analogues in the mammalian cells the bands for cytochromes a and b being replaced by a and b_1 respectively while the bands for cytochrome c and a_3 have not been demonstrated

At present it is not clear how closely the two cytochrome systems are related and how the results obtained in the experiments with *E coli* might be interpreted in the light of data accumulated for the cytochrome system in mammalian cells

DR S SASLAW (*Ohio State University*) *There are two points which I would* like to comment on Certainly I would agree wholeheartedly with Dr Woodward s paper and Dr Parker on the bone marrow situation with reference to chloramphenicol There undoubtedly have been some cases of aplastic anemia but in surveying the cases coming into the Ohio State

Medical Center with all the cases we see there, there has not been an increase in aplastic anemia since the advent of this drug So, maybe these people would have done the same thing with butazolidine thiouracil aminopyrine etc *In fact we have monkeys that we have had on dosages up to* 300 mg per kilo as long as 15 months without any change in the peripheral and bone marrow situation Another point I want to mention with reference to therapy which may sometimes get us into trouble is the cortisone adjunct I think we are all aware of the fact that with pathologic hyperadrenalism Cushing s disease we can figure anywhere from 66 to 40 some odd percent will show infection or delayed wound healing Also we are all aware of the experimental data with bacterial viral and fungal agents and the increased susceptibility to them in the presence of cortisone We are also aware that we are getting tuberculous processes in other infections breaking through because they have been masked while getting steroid therapy for other diseases So I would also like to quote Garrett when he says that cortisone in rickettsial diseases actually acts like a super aspirin I personally if I had the disease would rather go for an extra day of fever and not have the euphoria and not have to take a chance on the untoward reactions

DR SABIN There is nothing to say about it except that the substance which might work chemotherapeutically would be expected to work much better chemoprophylactically We have always thought that by the time we recognize the clinical manifestations of a virus infection it might be too late for chemotherapy even if you had an agent that would interfere with its multiplication On the other hand some of the diseases caused by the members of the lymphogranuloma venereum group do not seem to follow this particular concept While I have this microphone I would like to ask Dr Woolley a question In showing the effects of pectin on influenza virus in the egg Dr Woolley indicated that the experimental set up consisted of adding the pectin to the egg and then putting the virus in In view of the model that he put on the lantern slide I wondered if he would expect that *the pectin would combine with the enzyme on the virus particle in* such a manner that if it were added to the virus first and the virus particles were then washed and then added to the egg that he would still expect to get the same result and did he do such an experiment?

DR WOOLLEY *Yes* Dr Sabin *we are very much aware of the possibility* that the pectin merely precipitated the virus We have long had evidence that this is not the explanation In fact if it were I would not have troubled you with the reaction mechanisms which were shown The interaction of virus and pectin is one which apparently forms a very loose and dissociable complex *It is only* necessary to dilute the solution and thus to reduce the concentrations of reactants in order to dissociate the complex The pectin

appears to form this very loose complex but is in no way a virus precipitating agent—quite the contrary

DR COMMONER It might be useful if I said a few words about our experience with thiouracil and tobacco mosaic virus Dr Bawden has already discussed some of his work which confirms the observations we first made some years ago In turn we have confirmed Dr Bawden s very interesting observation that formation of lesions occurs in *N tabacum* plants when thiouracil is present It might also be useful to summarize here what we now know about inhibition of TMV biosynthesis by analogs of nucleic acid components Most of this work has been done by Dr Frank Mercer at the St. Louis College of Pharmacy After screening a large number of nitrogen base analogs it was found that thiouracil thiocytosine and thiothymine are by far the most active inhibitors These inhibitions were reversed only by uracil In other words uracil metabolism appears to be the phase of TMV biosynthesis most sensitive to analog inhibition Thiocytosine and thiothymine are in a sense also analogs of uracil From recent studies of nucleic acid changes in infected leaf we also find that the uracil required for TMV synthesis is in relatively short supply Thus uracil which is the distinctive nitrogen base of pentose nucleic acids (such as that of TMV) appears to represent a uniquely critical aspect of TMV reduplication

DR R T PARKER (University of Maryland) I would just like to say a few words in response to Dr Saslaw s comment regarding cortisone First of all limitations of time prohibited describing the dosage and duration of therapy but I think you may recall from the slide which was shown the duration of cortisone therapy in patients acutely ill with Rocky Mountain spotted fever as well as these other rickettsial diseases was extremely short—20 to 48 hours The dosage for that period of time is not excessive I don t think that we have to worry about tuberculosis or failure of healing when therapy is of such short duration Otherwise if it were for long periods of time I think that the comments would be well taken Personally in studying the patients included in this series as well as those that we have studied since that time we are pretty well convinced it may make the difference between life and death in late severely ill patients—and I emphasize late severely ill patients